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STANDARD METHODS
OF THE
DIVISION OF LABORATORIES AND RESEARCH
OF THE
NEW YORK STATE DEPARTMENT OF HEALTH

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③ laboratory animals
④ chemical analysis
⑤ culture media
⑥ laboratory glassware
⑦ diagnostic laboratories
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⑪ vaccines ⑫ antitoxins
② analytical methods

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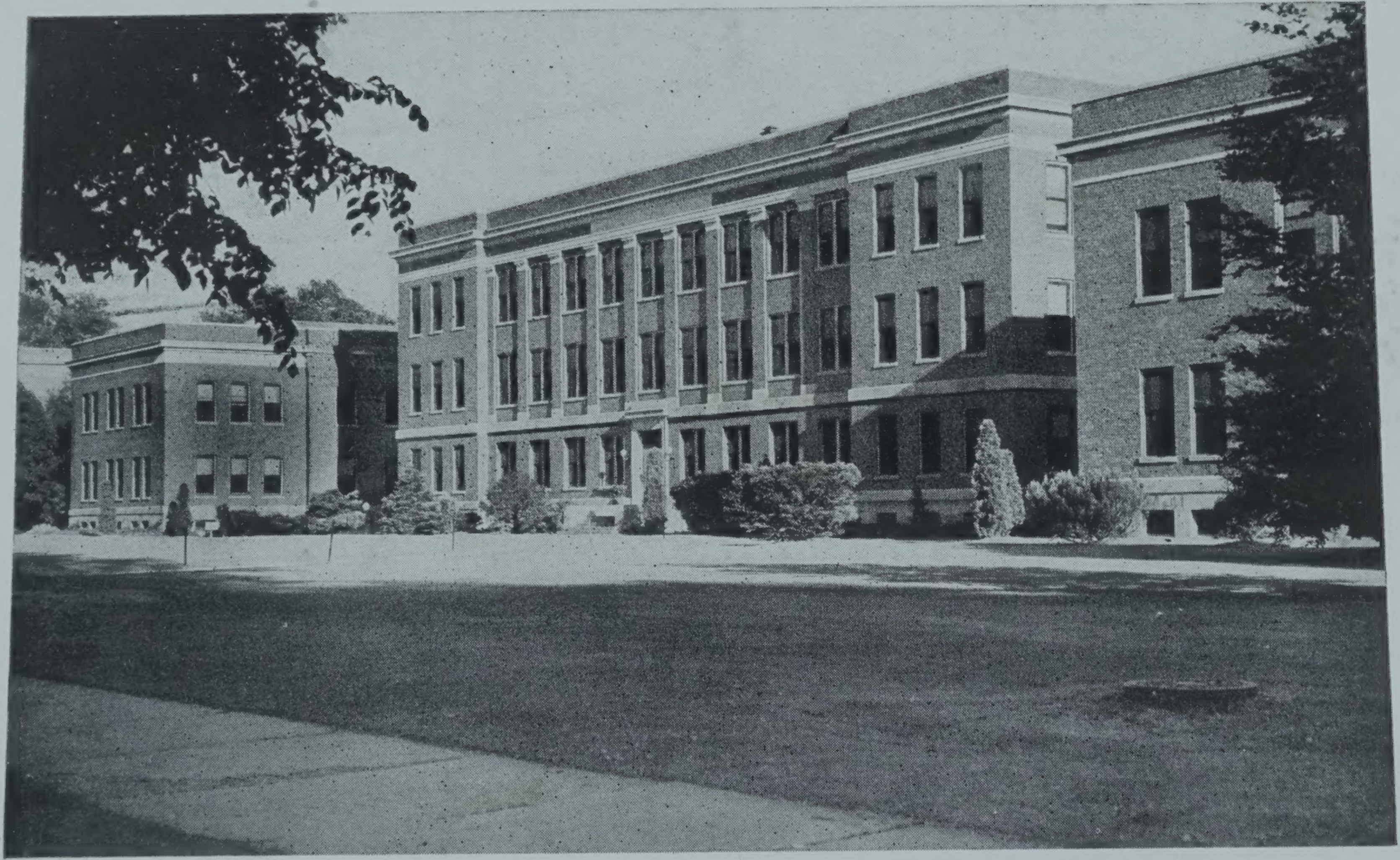

DIVISION OF LABORATORIES AND RESEARCH

North Façade Main Building, East and West Wings, New Scotland Avenue, Albany. Main building occupied 1919; East Wing, Diagnostic Laboratories, 1924; West Wing, Antitoxin, Serum, and Vaccine Laboratories, 1929; South Wing, Media Department, and Library, 1939.

STANDARD METHODS
OF THE
DIVISION OF LABORATORIES AND RESEARCH
OF THE
NEW YORK STATE DEPARTMENT OF HEALTH

AUGUSTUS B. WADSWORTH, M.D.

With a Foreword by
GILBERT DALLDORF, M.D.

THIRD EDITION

CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE,
LIBRARY
* MYSORE. *

LONDON
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Dedicated

To the Memory of
T. MITCHELL PRUDDEN
and
HERMANN M. BIGGS
Pioneers and Leaders in
Public Health

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FOREWORD

The important place which "Standard Methods" occupies is shown by the testimony of visitors and correspondents from many parts of the world. Older editions have been interleaved with translations in foreign languages and worn out in service. There is no better evidence of the usefulness of any book nor better justification for the present edition.

New knowledge continuously expands the field of public health and the public health laboratory. The original interest in pathogenic bacteria is now being extended to the viruses and challenged by the importance of neoplastic and degenerative diseases, nutrition, and such special problems as the development of blood and transfusion services. Chemistry has forged to the front and extensive fundamental studies of biologic relationships are obviously needed. The problem of forensic medicine has many ramifications which involve public health laboratories as they are organized in New York.

The principles of organization and study and the methods developed by Doctor Wadsworth and described in this volume will prove of equal value whatever future developments may be. They are principles which deserve the allegiance of all laboratory workers, whose obligations to him it would be difficult to exaggerate. I am happy to have an opportunity of personally acknowledging that obligation.

GILBERT DALLDORF.

PREFACE TO THE THIRD EDITION

This third edition of Standard Methods records the revisions and additions made from 1939 to January 31, 1945, the date of my retirement. In a few instances procedures then being studied have since been completed and included. This edition, therefore, marks the conclusion of thirty-one years of collaborative effort. Under the direction of Dr. Gilbert Dalldorf, a second period of further development begins auspiciously.

Changes will be noted in practically all sections of the book. Changes in procedure that are suggested from time to time in the course of the work are reviewed by a standing committee of the staff before they are referred to the director for approval. The review of all methods and the preparation of the revised manuscript have been shared by practically the entire staff working with the heads of departments. A complete revision has been made in the routine methods for the serodiagnosis of syphilis. An outstanding change is the substitution of the new antigen composed of cardiolipin, lecithin, and cholesterol for the cholesterolized alcoholic extract of beef heart that has been in general use for many years. The improved method for the preparation of the cardiolipin antigen is also given. The difficulties that beset the standardization of the complement-fixation test for syphilis illustrate the problem of adapting it to different diseases. It appears to offer, however, a most significant diagnostic aid, notably in the virus diseases, and the experience and researches of the past several years will provide a fundamental basis for the new applications. Certain methods in the fields of biochemistry and biophysics have been revised and new procedures added because they have become essential in many phases of the work. New also in this edition is a chapter on biologic assay.

The laboratory has experienced the manifold problems and emergencies of two world wars. During the first its contributions were directed chiefly to the training of technical personnel for the army laboratories and the distribution of surplus antitoxins and sera. During the second war the contributing services have been the release of trained staff to the limit of safety, and serologic examinations for the military services. The labor turnover in both wars exceeded 100 per cent at times but these difficult situations were met without lowering standards of service.

In the present war period emphasis has been placed on scientific research to an unparalled extent, and medical science, especially in its practical applications, has advanced beyond all expectations. Decades of basic research provided clearer concepts of the nature of disease processes and thus led to more specific methods of diagnosis, prevention, and treatment.

The viruses have now extended the field so long held by the bacteria. Chemotherapy has supplanted serum therapy in certain bacterial infections and supplemented it in others. In the last analysis, however, it is the protective mechanisms of the host that destroy the parasite. All lines of investigation thus lead ultimately to the biologic functions of host and parasite. Fortunately this laboratory through its research activities, which have been so continuously supported by successive commissioners of health, governors, and legislatures, has an experienced staff and the facilities to go forward. A laboratory of this scope and responsibility must go forward or backward; it cannot stand still. It must have the full support and confidence of those in authority.

No line can be drawn between academic and applied research. The practical problems, however, are not only a stabilizing control but afford opportunities for fundamental approaches to their solution and also extend the significance of the results. The training and experience of the staff in developing the practical methods presented in this book are assurance for the future work of the laboratory, especially with the guidance of Doctor Dalldorf who has been so actively identified with the newer fields, notably virus infection and the cellular metabolic activities of nutrition.

It seems appropriate to review briefly the organization under which these methods have been formulated. The present organization of the laboratory is the result of practical experience in the development of the work over a long period. It was based upon certain broad principles and policies with clearly defined objectives which have survived and appear sound, namely, maintenance of efficient extramural and intramural services and of research and investigation by the staff in assigned branches of the work. These objectives entailed system and order throughout, educational training and experience for the workers through responsibility in their assignments, and the provision of operational facilities through defined channels to avoid unnecessary delay and to fix responsibility. The organization is planned to relieve the scientific staff of routine administrative duties, within practical limitations. This in turn has broadened their responsibilities, added to the interest, and provided opportunities for each individual to develop according to his ability and experience. Development of the worker through responsibility in his assignment is the most direct approach to high standards of work.

The chart of organization indicates the main divisions and responsibilities in the work. Certain general services were early established. They have been an essential economy and have provided opportunities for the training of experienced workers and for developing research in all branches of the work. The first of these groups to take form was the library, now a comprehensive specialized reference collection. Responsibility for the editorial and publications work, for the care of permanent records of research, and for the administration of the museum is also in the library department. Although the scope of the original plan for this group has not changed essentially, the services have developed in a remarkable manner as the need has grown from year to year. A second general service was developed as a result of centralizing the preparation of media and glassware. Practically all new appointees spend a short period in this department before undertaking their particular assignments. Technicians acquire a basic training here and advanced workers, first-hand information in regard to the facilities available. The maintenance and distribution of laboratory animals is another general service, associated with the antitoxin, serum, and vaccine laboratories because of their major demands. In charge of experienced veterinarian-bacteriologists, it safeguards standards of animal work and provides opportunities for the study of animal pathology and epizootics. The collection of type cultures has always been considered part of the museum. It serves all departments, however, and for practical purposes operates as a separate unit, providing basic training in systematic bacteriology for new appointees and thus ensuring a uniform technic. Other services have been developed as needed, for example, in biochemistry, and biophysics. These specialized general service groups have, in general, an independent status but work in close touch with and are responsible to the director or the associate director. The scientific staff of the director constitutes a special group that is in close touch with all the investigative work of the laboratory. Research is distributed wherever it can be most effectively done by qualified workers, whether they happen to be assigned to the routine services concerned with the problem or elsewhere in the organization. Any member of the staff may submit suggestions or an outline for experimental work. These phases of the organization provide valuable educational opportunities for the training of the staff of a large institution.

The administrative offices have full responsibility for the fiscal and other business phases of the work of the laboratory. Each of the main departments has a secretary who is responsible for the office workers assigned to it. These secretaries, with the secretary to the director and the librarian, form the Secretariat which is responsible to the director and has the important function of coordinating the various routine and scientific activities of the Division. The Secretariat submits reports to the director and the Laboratory Committee. The Laboratory Committee under the chairmanship of the associate director is the central unit that administers the laboratory in general. The transactions of this Committee and reports of the Secretariat are reviewed at the monthly Conference of the Director. Other intramural committees of the laboratory by which responsibility has been distributed and representation broadened and which were made increasingly necessary by the growth of the staff to more than four hundred are the Committee on In-Service Training; Efficiency Rating Committee; Executive Committee of the Seminar; Committee on Standard Methods; Committee on Laboratory Equipment and Supplies; Accident Prevention Committees; Laboratory Staff Association; Staff Personnel Committee.

Thus it is that the organization of the laboratory based upon a few simple but fundamentally sound principles and objectives has been developed in detail, with standard methods to facilitate its operation and provide for changes without the sacrifice of objectives. Some such organization is vital in a scientific institution with a large staff and many and varied activities. In this instance the plan adopted has proved an essential safeguard to the broader interests and objectives.

The growth of the staff has paralleled that of the organization. Numbering seventeen in 1914, it had increased to 175 in 1919 when the first of the present buildings was occupied. Of these, forty still remain. This nucleus has been invaluable in maintaining uniformity and continuity in policies and methods. It has helped to inspire a singleness of purpose that is unique in an institution of this character and size, while new members have contributed fresh vigor and new outlooks. Indeed it is the spirit of the whole staff that accounts for whatever merit this volume possesses.

AUGUSTUS B. WADSWORTH.

June, 1946.

PREFACE TO THE SECOND EDITION

The preface and introduction to the first edition of Standard Methods are retained because they are still essentially correct. Dr. Edward S. Godfrey, Jr., the present Commissioner of Health, has continued the policies of his predecessors in regard to laboratory service for the State and, through his effective support, has extended the scope and character of the work. As in the previous edition, the preparation of these methods for publication has been the task of the entire staff in collaboration with the heads of the several departments.

The changes reflected in these so-called "standard" methods are incident to the expansion of the central laboratories and the extension of the general laboratory service rendered the State. The number of laboratories approved for diagnostic service has increased to 128, the qualifications of directors, pathologists, and bacteriologists in charge of approved laboratories have been advanced, and 56 laboratories have been approved for the sanitary examination of milk and water; the collaboration between the local laboratories and the central state laboratories has been developed, strengthened, and made more effective; and the staff of the division has been augmented during the years of this expansion so that it now numbers approximately 475. Besides the director and associate director, there are two assistant directors in charge, respectively, of the antitoxin, serum, and vaccine, and the diagnostic laboratories, pathologists, bacteriologists, chemists, sanitary chemists, physicists, veterinarian-bacteriologists, and assisting staff of all grades.

This second edition differs from the first largely because of the impracticability of repeating all the meticulous detail and at the same time including important new material in the space of one volume. It is hoped that the revision has been accomplished without sacrificing the objective of an adequate description of the essential procedures. This, however, could only be done by segregating in one section on general laboratory procedures the technical details of methods common to different branches of the work. The reader is therefore put to some inconvenience by the cross references inevitable in such an arrangement. Under the special chapters these general technics are described sufficiently for the experienced worker, but the novice and the less experienced technician should become thoroughly familiar with the introductory chapters before following the methods of the diagnostic or the antitoxin, serum, and vaccine laboratories.

There have been many changes in procedure. Particular mention may be made of the introduction of the new quantitative technic in the complement-fixation tests, the revised colloidal gold test, and of new methods in the production, concentration, and standardization of certain therapeutic sera.

Basically, the laboratory is concerned with the problems of infection and immunity, the investigation of which has so broadened that departments of biochemistry and biophysics have been developed since the earlier edition. The microanalytical methods of Pregl have been adapted to the requirements of special problems in research, as have also biochemical methods; they can be included only in the briefest outline, with references for those who have not had practical experience in these particular fields. The applications of biophysical analysis, such as quantitative absorption spectrophotometry, electrophoresis, and electrical conductivity, have not been included, although they are in use and have been indicated in published research. The technic of tissue culture has been omitted but it also has been developed as an aid to special investigations, some of which have been reported.

The publication of this second edition marks the twenty-fifth anniversary of the Director, of Dr. Mary B. Kirkbride, Associate Director, and of Dr. Ruth Gilbert, Assistant Director. The collaboration that had its beginnings in the laboratories of the College of Physicians and Surgeons of Columbia University has thus been continued throughout the period of public service for New York State. It is fitting to record that this uninterrupted adherence to a common purpose has been responsible in no small measure for the advances in public health laboratory services in the State, local as well as central, and for instituting the technical procedures that are recorded in this volume.

AUGUSTUS B. WADSWORTH.

May, 1939.

PREFACE TO THE FIRST EDITION

The reorganization in 1914, and subsequent development of the laboratory and its present staff as a branch of a large department of the state government was possible only with the sympathetic and appreciative support of Dr. Hermann M. Biggs, Commissioner of Health for nearly ten years, and of Dr. Matthias Nicoll, Jr., Commissioner for the past three years. The governors of the state and the leaders of the legislature, during this period, have also contributed in no small measure the means with which the work has been done. The laboratory has thus been free to concentrate upon its scientific pursuits without political interference. Finally, the Civil Service Commission has always cooperated to advance standards in personnel.

Laboratory service for all districts of the state outside the Greater City of New York was early considered in the scheme of organization and has proceeded, step by step, according to a preconceived plan. The establishment of branches of the central state laboratory was considered, on the one hand, and on the other, the development of local laboratories supported and operated independently but under supervision. The one promised immediate results but involved a large expenditure on the part of the state, and a service which would never become adequate in scope for all rural districts, whereas the other, although beset with many pitfalls and difficulties at every turn, relieved the state budget, stimulated local initiative, interest, and pride, and offered the opportunity in its successful development of providing an adequate service for all districts. Thus it was that only one branch of the main central laboratory was established, in New York City, for the twofold purpose of covering an inaccessible, unserved district and, at the same time, bringing the state laboratory work into close touch with the educational and hospital facilities of the great medical center in New York City.

Attention was therefore directed to the consolidation of the existing local laboratories, the advancement of standards in personnel and methods of work, and at the same time, to the problem of, so to speak, weaving these heterogeneous laboratory units into a homogeneous laboratory service for the state as a whole with uniform standards of work.

The advancement of standards started with the issuance of an approval after inspection, and has now reached the stage of advanced requirements as to the qualifications of the personnel, inspection of facilities and equipment, agreement as to methods of technical and office procedure, periodic testing of the results of the work—thus, complete supervision as to the fulfillment of minimum requirements of approved laboratories. The confidence and cooperation of these local laboratories were secured by embracing every opportunity of rendering valuable service to them, and recognizing and supporting, in every way possible, their independence, with only the one provision that standards of work were maintained. Owing to the whole-hearted support of the local laboratories, this program has progressed with the most gratifying results, almost on its own initiative, because it eliminated incompetent and unreliable laboratories and is now culminating most successfully, through the development of the activities of the New York State Association of Public Health Laboratories, in which organization every laboratory, including the state laboratory, has only one authorized vote on questions of policy; and through the increased facilities which have become available since grants of state aid have been made to the municipal and county laboratories. Thus it is that there are now 105 approved laboratories serving the state, and the standardization of laboratory procedure has become one of the paramount interests of the central state laboratory.

These standard methods, however, are not established by law but are subject to constant revision. Whatever official significance they may connote pertains only to laboratory activities in the state exclusive of the Greater City of New York. Federal standards promulgated by the United States Government rest upon authority to control interstate commerce and relate chiefly, if not exclusively, to the sale of biologic products.

The methods of this laboratory are the result of long experience in dealing with a large heterogeneous staff whose qualifications, on entering the organization, have ranged from mere high-school training to thorough preparation in scientific subjects following fundamental academic education. Early in the history of the laboratory, it became evident that a high standard of work could only be maintained by providing each worker with precise, detailed directions for his routine technical work. These directions, constantly improved throughout more than a decade by contributions from the staff, constitute this volume.

Quite apart from immediate practical aims, however, the development of these standard methods has served a much deeper and broader purpose. The laboratory is engaged in performing daily a large volume of routine work: but it is also a laboratory for research, since research is essential to the advancement of routine standards. How then to develop in the staff the capacities, the vision, and the genius so essential in research, early became a problem in advanced education in an institution primarily without educational scope or purpose. Whatever measure of success has been achieved in solving this problem must be attributed fundamentally to the working out in detail of these standard methods. It has been required that the literature bearing on all proposed scientific investigations should be carefully reviewed, and the purpose, scope, and plan of study of such investigations should be approved before any work might be undertaken, the result of which might form the basis for changes in the methods. In this way, the necessary stimulus has been provided which has led to the inception and growth of the spirit of research. The results have been so fruitful that the laboratory now has in its scientific file a collection of data of incalculable value to its work, while the educational effect of the policy adopted has been immediate and cumulative.

Any criticism of the material as a whole, or of any of its sections, will be received with appreciation. The methods, which incidentally are a record of the laboratory's progress up to the present time in formulating precise procedures for the conduct of the work, are published for a threefold purpose: to keep uniform the established methods; to instruct the new worker and prescribe exact procedures for him and for those who are unable to take independent responsibility; and to serve as a guide to the policy which the trained, responsible worker may follow or depart from as the immediate situation demands.

INTRODUCTION TO THE FIRST EDITION

During the past ten years, these methods, which have been in typewritten form, have been so continually revised and improved that printed copies are now necessary not only for further revision but to meet the requests for copies from other laboratories engaged in similar work. The extent of the detail is the result of experience in guarding against error. The arduous task of preparation for publication has been shared by the entire staff although the chief responsibility has been assumed by the heads of the laboratory departments. Each series of methods is a unit in itself, and does not necessarily conform to the others in arrangement or in the amount of detail it contains.

The instructions and directions for the media and glassware groups, for instance, are precise and somewhat elementary because the methods are intended for workers who, at first, as a rule, are unfamiliar with laboratory regulations. The diagnostic methods are equally precise but they are focused differently, being intended for workers with some experience in laboratory technic. The methods for the examination of water, sewage, ice, and milk in the laboratories for sanitary and analytical chemistry are practically the same as those prepared by the American Public Health Association, and have merely been slightly modified to meet special conditions. They are included with the other methods principally because any presentation of the work done in this laboratory would be incomplete without them, and because they serve as a guide to the newcomer. The formulation of the methods used in the antitoxin, serum, and vaccine laboratories has presented a series of problems. Owing to the lack of definite knowledge concerning the nature and interaction of the bacterial poisons and their antitoxins or antisera, there is a great deal of empiricism in laboratory practice in this field; and the results of research, which has everywhere been directed to the study of prophylactic and therapeutic agents, have necessitated repeated modifications of the methods prescribed.

The administrative and the secretarial and library groups have been organized and developed in accordance with the best office and library practice. In the present volume, their functions in the laboratory organization are briefly but definitely outlined, and information which it is essential for the scientific staff to have concerning the work of these groups is given in detail.

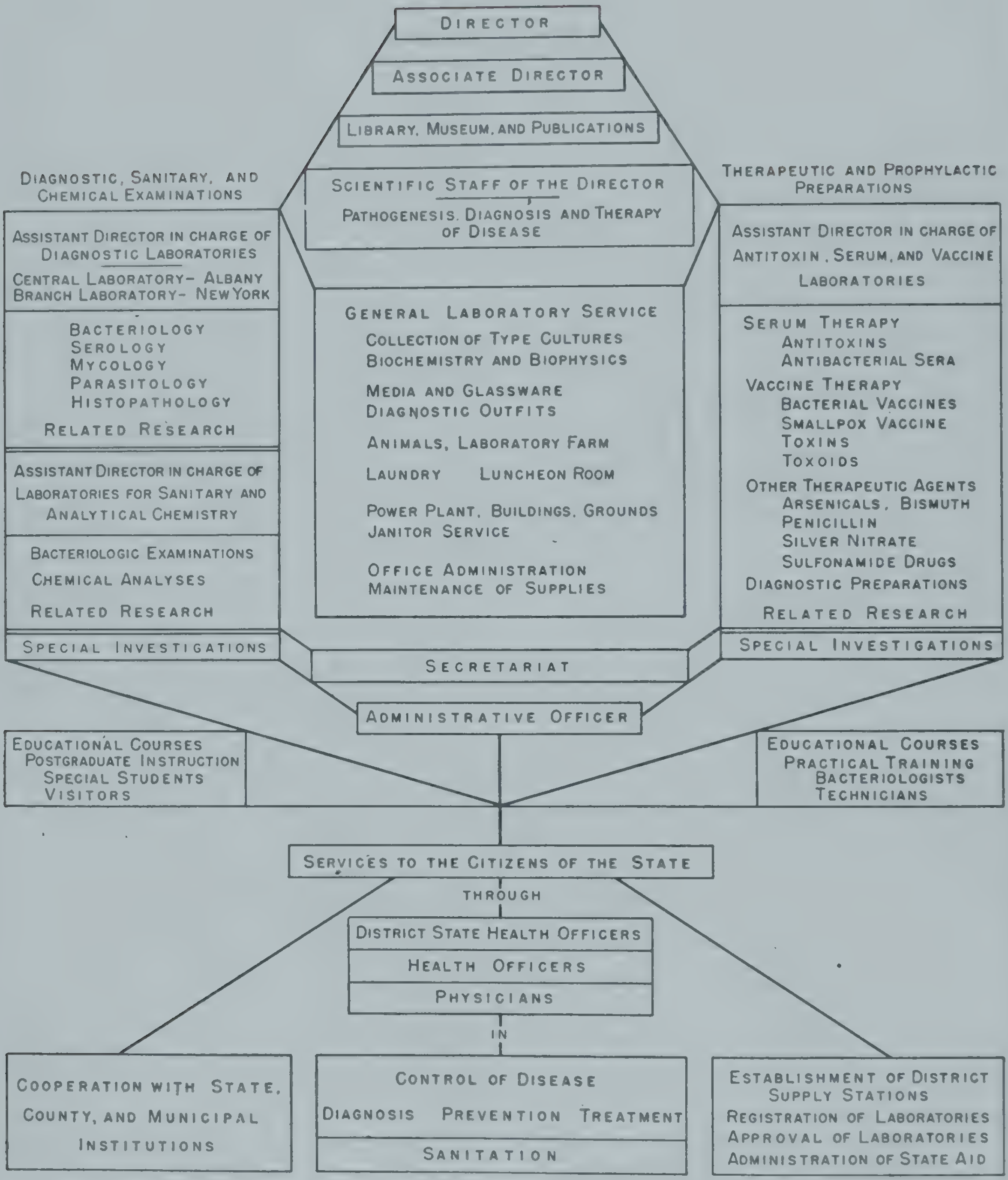
The organization of the Division of Laboratories and Research is best presented in a diagram which shows how the laboratory is administered and the various activities are correlated. Apart from the cleaners, laborers, and other general workers required to maintain any institution, the strictly scientific and technical personnel is separated into two main divisions: one making the necessary examinations and analyses; and the other preparing biologic and medicinal products. The general service needed by these two is supplied by a third division composed of several groups. They prepare the media, glassware, and reagents and carry out the work connected with sterilization, and with certain laboratory tests.

This third division of general service groups includes besides, a number of secretaries, stenographers, and clerks in a main office under the direction of a competent executive who not only coordinates and correlates the general work of the laboratory, but whose department is also, to a large extent, the channel of communication between the laboratory and those outside. Finally, as part of the general service available for members of the two main divisions, the laboratory has a well-trained library staff who not only administer the library material, but also file and index all outlines and reports on research written by members of the staff (the Scientific File). Likewise, they help workers in every way to prepare their papers for publication.

The work of a public-health laboratory is thus not only scientific and technical, but business-like. The clerical staff serves the professional workers, and at the same time exercises a most important control, insuring accuracy, promptness, order, and system. It may be added that while the clerical staff is distinct from the scientific staff, yet whatever the educational background of its members may be, they require special training and experience in a public-health laboratory in order to do the work well and quickly, and they must be infused with the spirit of research and responsibility to maintain standards of clerical service in keeping with the high aims of scientific work. Every facility in the way of standard methods, adequate clerical, secretarial, and library service is thus provided for the members of the scientific staff to the end that they may devote their best energies to the continual improvement of their work.

Besides the director and the assistant director, the staff includes at present 5 bacteriologists, 1 pathologist, 5 chemists, 1 veterinarian-bacteriologist, 15 assistant bacteriologists, 2 assistant serologists, 2 assistant pathologists, 9 assistant chemists, 12 laboratory assistants, 4 laboratory apprentices, 52 technical assistants and helpers.

The appropriations for 1926–1927 include $368,660.00 for personal service, $166,220.00 for maintenance and operation, and $100,000.00 for state aid for approved laboratories.


DIRECTOR
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DIAGNOSTIC, SANITARY, AND CHEMICAL EXAMINATIONS
ASSISTANT DIRECTOR IN CHARGE OF DIAGNOSTIC LABORATORIES
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BRANCH LABORATORY– NEW YORK
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BACTERIOLOGIC EXAMINATIONS
CHEMICAL ANALYSES
RELATED RESEARCH
SPECIAL INVESTIGATIONS
GENERAL LABORATORY SERVICE
COLLECTION OF TYPE CULTURES
BIOCHEMISTRY AND BIOPHYSICS
MEDIA AND GLASSWARE
DIAGNOSTIC OUTFITS
ANIMALS, LABORATORY FARM
LAUNDRY
LUNCHEON ROOM
POWER PLANT, BUILDINGS, GROUNDS
JANITOR SERVICE
OFFICE ADMINISTRATION
MAINTENANCE OF SUPPLIES
THERAPEUTIC AND PROPHYLACTIC PREPARATIONS
ASSISTANT DIRECTOR IN CHARGE OF ANTITOXIN, SERUM, AND VACCINE LABORATORIES
SERUM THERAPY
ANTITOXINS
ANTIBACTERIAL SERA
VACCINE THERAPY
BACTERIAL VACCINES
SMALLPOX VACCINE
TOXINS
TOXOIDS
OTHER THERAPEUTIC AGENTS
ARSENICALS, BISMUTH
PENICILLIN
SILVER NITRATE
SULFONAMIDE DRUGS
DIAGNOSTIC PREPARATIONS
RELATED RESEARCH
SPECIAL INVESTIGATIONS
SECRETARIAT
ADMINISTRATIVE OFFICER
EDUCATIONAL COURSES
POSTGRADUATE INSTRUCTION
SPECIAL STUDENTS
VISITORS
EDUCATIONAL COURSES
PRACTICAL TRAINING
BACTERIOLOGISTS
TECHNICIANS
SERVICES TO THE CITIZENS OF THE STATE
THROUGH
DISTRICT STATE HEALTH OFFICERS
HEALTH OFFICERS
PHYSICIANS
IN
COOPERATION WITH STATE, COUNTY, AND MUNICIPAL INSTITUTIONS
CONTROL OF DISEASE
DIAGNOSIS
PREVENTION
TREATMENT
SANITATION
ESTABLISHMENT OF DISTRICT SUPPLY STATIONS
REGISTRATION OF LABORATORIES
APPROVAL OF LABORATORIES
ADMINISTRATION OF STATE AID

FIG. 1. CHART OF ORGANIZATION

DIVISION OF LABORATORIES AND RESEARCH[1]

Gilbert Dalldorf, M.D., *Director*[2]

Mary B. Kirkbride, Sc.D., *Associate Director*

Scientific Staff of the Director

Grace M. Sickles, *Associate Bacteriologist*
Elizabeth Maltaner, *Associate Bacteriologist*
Frank Maltaner, Ph.D., *Associate Biochemist*
Irving Gordon, M.D., *Senior Medical Bacteriologist*[3]
Myrtle Shaw, Ph.D., *Senior Bacteriologist*
Mary C. Pangborn, Ph.D., *Senior Biochemist*
Christine E. Rice, Ph.D., *Assistant Bacteriologist*
Rena L. Dodd, *Assistant Bacteriologist*
——————————, *Assistant Bacteriologist*
John Kent, *Junior Bacteriologist*[4]
——————————, *Junior Bacteriologist*

Lilian C. Smith, *Secretary to the Director*

General Laboratory Service

Collection of Type Cultures:	Julia M. Coffey, *Associate Bacteriologist*
Biochemistry:	Jaques Bourdillon, M.D., *Senior Medical Biochemist* Rachel Brown, Ph.D., *Senior Biochemist* William R. Thompson, Ph.D., *Senior Biochemist* James J. Quigley, *Assistant Biochemist* Frances Gillum, *Assistant Biochemist* Lucena K. Robinson, *Assistant Bacteriologist*
Biophysics:	Marguerite O'L. Crowe, *Senior Biochemist* Amy Walker, *Assistant Biochemist*
Media and Glassware:	Mary W. Wheeler, *Associate Bacteriologist* Edward Green, *Senior Laboratory Worker*

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[1] September 1, 1945. The total staff numbered 430.

[2] Appointed Director April 16, 1945. Augustus B. Wadsworth, M.D., appointed Director February 9, 1914, retired January 31, 1945.

[3] On war leave.

[4] On military leave.

Antitoxin, Serum, and Vaccine Laboratories

Harold W. Lyall, Ph.D., *Assistant Director in charge*
John K. Miller, M.D., *Associate Medical Bacteriologist*[5]
Charles A. Griffin, D.V.M., *Associate Veterinarian-Bacteriologist*
Sophia M. Cohen, *Senior Bacteriologist*
Jessie L. Hendry, *Senior Bacteriologist*
Gretchen R. Sickles, *Senior Bacteriologist*
Philip P. Murdick, *Senior Biochemist*
Helen R. Odell, *Assistant Bacteriologist*
Betty E. Laros, *Assistant Bacteriologist*
Donald J. Dean, D.V.M., *Assistant Veterinarian-Bacteriologist*

Katherine M. Shelley, *Secretary*

Diagnostic Laboratories

Ruth Gilbert, M.D., *Assistant Director in charge*
Fred W. Stewart, M.D., *Principal Diagnostic Pathologist*[6]
Joseph Schleifstein, M.D., *Associate Diagnostic Pathologist*
Albert H. Harris, 2d, M.D., *Associate Medical Bacteriologist*[7]
Carl Lange, M.D., *Associate Bacteriologist*
——————————, *Senior Diagnostic Pathologist (Parasitology)*
Marion B. Coleman, *Senior Bacteriologist*
——————————, *Assistant Medical Bacteriologist*
Thelma Muraschi, *Assistant Bacteriologist*
——————————, *Assistant Bacteriologist*
Mildred S. Wilson, *Junior Bacteriologist*
Mary E. Clark, *Junior Bacteriologist*
Gladys Gnesh, *Junior Bacteriologist*
Louise Elmer, *Junior Bacteriologist*
Emanuel Tahler, *Junior Bacteriologist*

Louise P. Main, *Secretary*

BRANCH LABORATORY, NEW YORK CITY

Edgar M. Maillard, M.D., *Associate Diagnostic Pathologist in charge*
Frank W. Foote, M.D., *Associate Diagnostic Pathologist*
Elizabeth L. Hazen, Ph.D., *Senior Bacteriologist*
Lise L. Graff, M.D., *Assistant Medical Bacteriologist*

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[5] On military leave.
[6] On part-time service.
[7] On military leave.

Henry Bartels, *Assistant Bacteriologist*
Anne E. Orzel, *Junior Bacteriologist*

Jennie Effron, *Secretary*

Laboratories for Sanitary and Analytical Chemistry
F. Wellington Gilcreas, *Assistant Director in charge*
Walter S. Davis, *Senior Sanitary Chemist*
Wallace W. Sanderson, *Senior Sanitary Chemist*
James E. O'Brien, *Junior Analytical Chemist*

Eleanor Byrne, *Secretary*

Library, Museum, and Publications Department
Anna M. Sexton, *Librarian*
Hester A. Wetmore, *Assistant Librarian*
Margaret M. Clark, *Department Librarian*
Dorothy S. Keck, *Department Library Assistant*
Frank C. Reed, *Laboratory Illustrator and Photographer*

Mary D. Morrissey, *Secretary*

Administrative Offices
Ila M. Dutton, *Administrative Officer*
Emma L. Dater, *Principal File Clerk*
Grace E. Keck, *Principal Clerk*

GENERAL LABORATORY PROCEDURES

CHAPTER 1

GENERAL BACTERIOLOGIC TECHNIC

Apart from the technical standards essential to prevent contamination of cultures and to minimize the danger of infection to the worker, it is almost axiomatic that in a laboratory where highly diversified bacteriologic investigations are undertaken the technic should be uniform.

Directions in the basic procedures for the isolation and cultivation of pure cultures and for the precautions to be observed in handling infective materials are therefore outlined and for convenience assembled in this chapter. Some of the technics may, obviously, be modified or adapted to meet particular needs.

BASIC INSTRUCTIONS

Laboratory coats are worn to protect the clothing. In all operations in which infective material might be spattered, protect the eyes with glasses or goggles. Whenever there is danger of spattering corrosive fluids, protect the entire face with a plastic shield; the hands and clothing, with gloves and aprons made of rubber or other resistant material. Wash the hands frequently and thoroughly with soap and water.

Whenever practicable, prophylactic vaccine is administered. For example, vaccination is required for workers who are assigned to studies of *Bacterium typhosum* or the viruses of variola or vaccinia.

All media, glassware, and instruments must be sterile. The procedures must be precise to prevent infection of the worker and to avoid contamination. Keep the working space clean and free from infective material by frequent washing with 1-per-cent cresol (*F75*). Handle cultures and specimens with care to avoid spattering. Keep cotton saturated with 1-per-cent cresol readily available to remove any drops that may fall.

Immediately after use, place all contaminated apparatus in containers for sterilization. Use covered containers unless the apparatus is to be immersed completely in disinfectant or placed immediately in the sterilizer. Avoid so loading the containers that the lids do not fit tightly or the weight is too great to place easily in the sterilizer. Place used pipettes in jars of 1-per-cent cresol (*F75*); glass slides and cover glasses used for moist preparations, in pans to which a 2- to 5-per-cent solution of sodium carbonate is added before boiling; other glassware, in pails of suitable size to be placed in the sterilizer; surgical instruments, in covered pans of water for boiling.

When accidents occur in which containers are broken and infective material spilled, collect broken glassware carefully to avoid cuts, and place it in a pail for sterilization. Wipe the working space and floor thoroughly with 1-per-cent cresol (*F75*) and wrap contaminated coats in paper for sterilization. Wash minor abrasions or skin exposed to infective or corrosive materials immediately under flowing tap water. If infective material is taken into the mouth, use at once and thoroughly the antiseptic mouthwash, see p. 28, that is available in every laboratory room. Report all accidents to the staff physician without delay so that injuries may be treated promptly and measures taken to prevent infection.

Highly Infective Material and Pathogenic Sporeforming Microorganisms.—Use special caution in handling highly infective material. Insofar as practicable, work with an assistant in a room fully equipped and assigned for the purpose. Wear special gowns and, when necessary, rubber gloves, goggles, and masks. Before these accessories are removed from the room, place them in containers for sterilization. When the examination is completed for the day, wash the working space and nearby floor with 1-per-cent cresol (*F75*).

When a needle or loop is used, heat with particular care to avoid spattering. When a pipette is required, use a safety pipette filler or a mouthpiece (see p. 8) and have the assistant open and close all containers. Hold the tip of the pipette over a Petri plate containing cotton saturated with 1-per-cent cresol to catch any drops that may fall. Store cultures and specimens either in locked cupboards or in containers on which the species of microorganism and the warning "Caution" are clearly marked in red pencil. Discard contaminated apparatus in metal containers and place them in a sterilizer. If space in a sterilizer is not immediately available, mark the containers "Do not touch" and leave in the workroom until they can be accommodated.

PREPARATION OF CULTURES

In the preparation of cultures, selection of the instrument for inoculation depends upon the medium and the amount of inoculum required. In general, a needle serves for the inoculation of stab cultures in solid or semisolid media and for fishing colonies. A loop (from 2 to 4 mm. in diameter) is used for inoculation of the surface of solid media, for transfer of small inocula from liquid or solid media to similar media, for fishing colonies of microorganisms that grow sparsely, and for the preparation of films for microscopic examination. A pipette is employed for transfer of relatively large or measured volumes of liquid or semisolid material. In addition to graduated and volumetric pipettes, "Asepto" syringes, or capillary pipettes prepared by drawing out glass tubing to provide tips of different diameters are convenient for transferring fluids. Such pipettes are usually controlled by rubber bulbs.

INOCULATION OF MEDIA

Immediately before subcultures are made, label each container of medium to be inoculated with the number of the culture or specimen and the date.

In general, work with one culture is completed before the containers for the next are labeled. Paper labels or tags that can be easily marked with a lead pencil are attached to each container in a position that will not interfere with observation of growth. Glassware that is used for intermediate procedures and Petri plates are marked with a wax pencil.

From each seed culture prepare a film and stain by Gram's method. If no evidence of contamination is found, place the seed culture in front of the containers to be inoculated and in a convenient position with relation to a lighted burner so that the operations may be performed without unnecessary motion. Compare the number on the seed culture carefully with that on each container to be inoculated. When the transfer is being made, hold the containers close to the flame in a well-slanted position; flame the mouth of each container after opening and before closing. Immediately before and after use, flame the needle or loop. In transferring material, avoid touching the necks of the containers. Take care that plugs are reinserted securely. When the containers are large, or so full that they cannot be slanted, have an assistant help with the transfer by removing the plugs, flaming the mouths of the containers, and holding the flame in a horizontal position above the opened mouth during the inoculation. When the transfer of one culture is completed, place the seed culture behind the containers of freshly inoculated media. Streak each seed culture on a plate of solid medium, and after incubation examine the growth for homogeneity. Make additional examinations whenever contamination is suspected.

With Needle or Loop.—To make subcultures from one tube to another with a needle or loop, support the two tubes together between the thumb and fingers of one hand—the seed tube next to the operator. In the other hand, hold the instrument like a pencil and sterilize by holding the wire vertically first in the cooler part of the flame and then along the cone until the wire turns dull red. Take care that the end of the handle to which the wire is attached is also flamed. Grasp the plug in the seed tube between the 3d and 4th fingers and remove it by slightly rotating the tube. Similarly remove the second plug, holding it between the 2nd and 3d fingers. (See figure 2.) Flame the mouths of the tubes; introduce the instrument of transfer into the seed tube, collect the culture and distribute it in the second tube. Flame the mouths of both tubes again, immediately replace the plugs in the same order and with the same rotary motion used for their removal, and sterilize the instrument. To inoculate larger containers, use a similar technic, but handle separately the seed culture and the container of medium that is to be inoculated.

When several tubes or other small containers of fluid medium are to be inoculated from one culture grown on solid medium, the following modified technic is convenient. Hold the container to be inoculated in a sharply slanted position and, with a loopful of culture, touch lightly the inner surface at a point that will be covered by the medium when the container is placed upright. With the same loopful of culture inoculate similarly the media in the other containers. Do not thrust the loop into the fluid medium. Handle each container separately and put aside all containers inoculated with one culture before proceeding to the next.

To inoculate Petri plates, raise and tilt the top just enough to admit the instrument so that exposure of the medium to microorganisms in the air is at a minimum.

With Pipette.—Remove and hold the lid from the can of pipettes by grasping it between the last two fingers and the palm of the hand; flame the mouth of the container; remove a pipette with the thumb and first finger without touching the other pipettes. (See figure 3.) Immediately flame the mouth of the container and replace the lid. Transfer the desired amount of inoculum by the procedure described for the loop, except that only one tube is handled at a time. Use a fresh pipette each time the seed tube is opened.

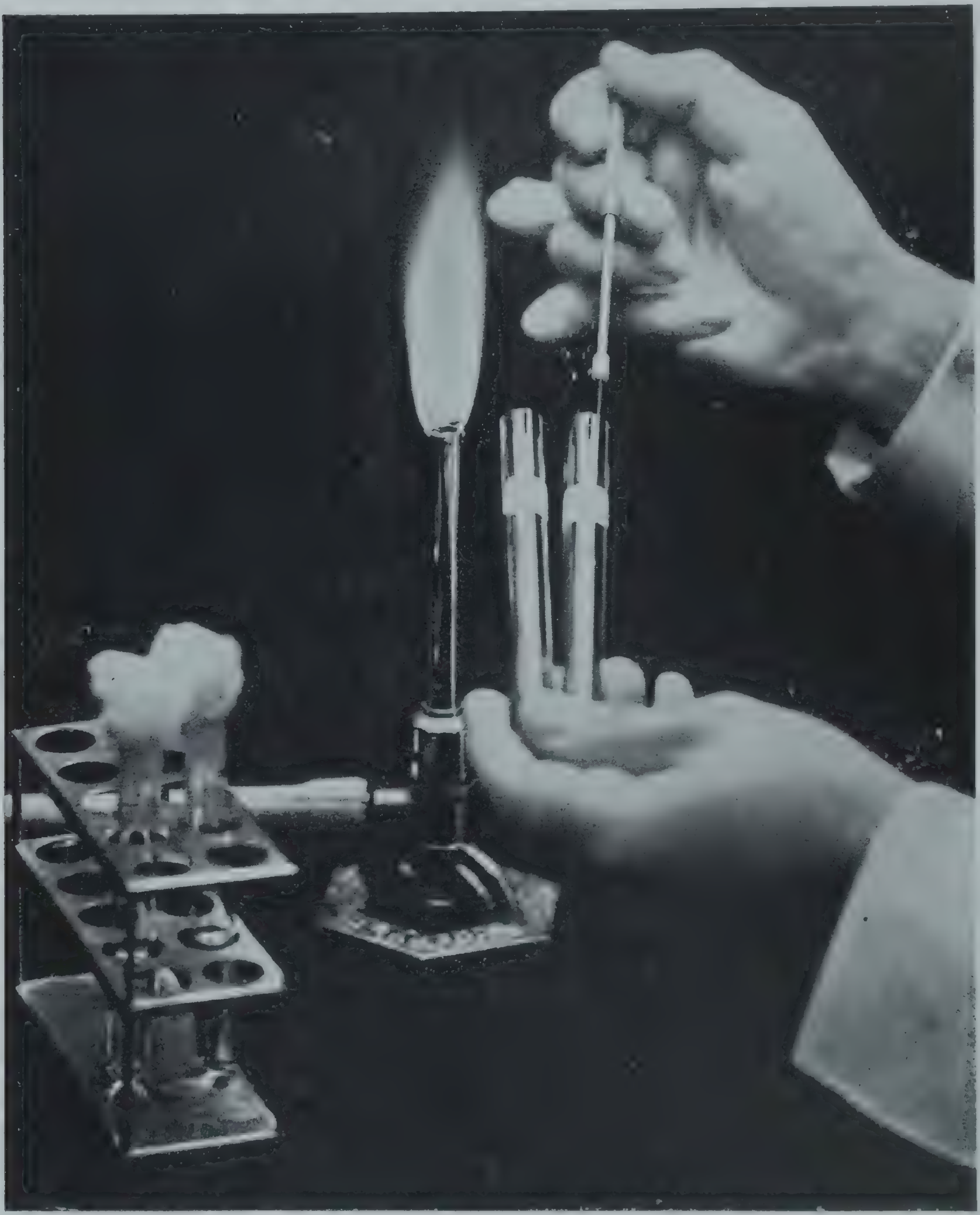

FIG. 2. TRANSFERRING CULTURE WITH WIRE LOOP

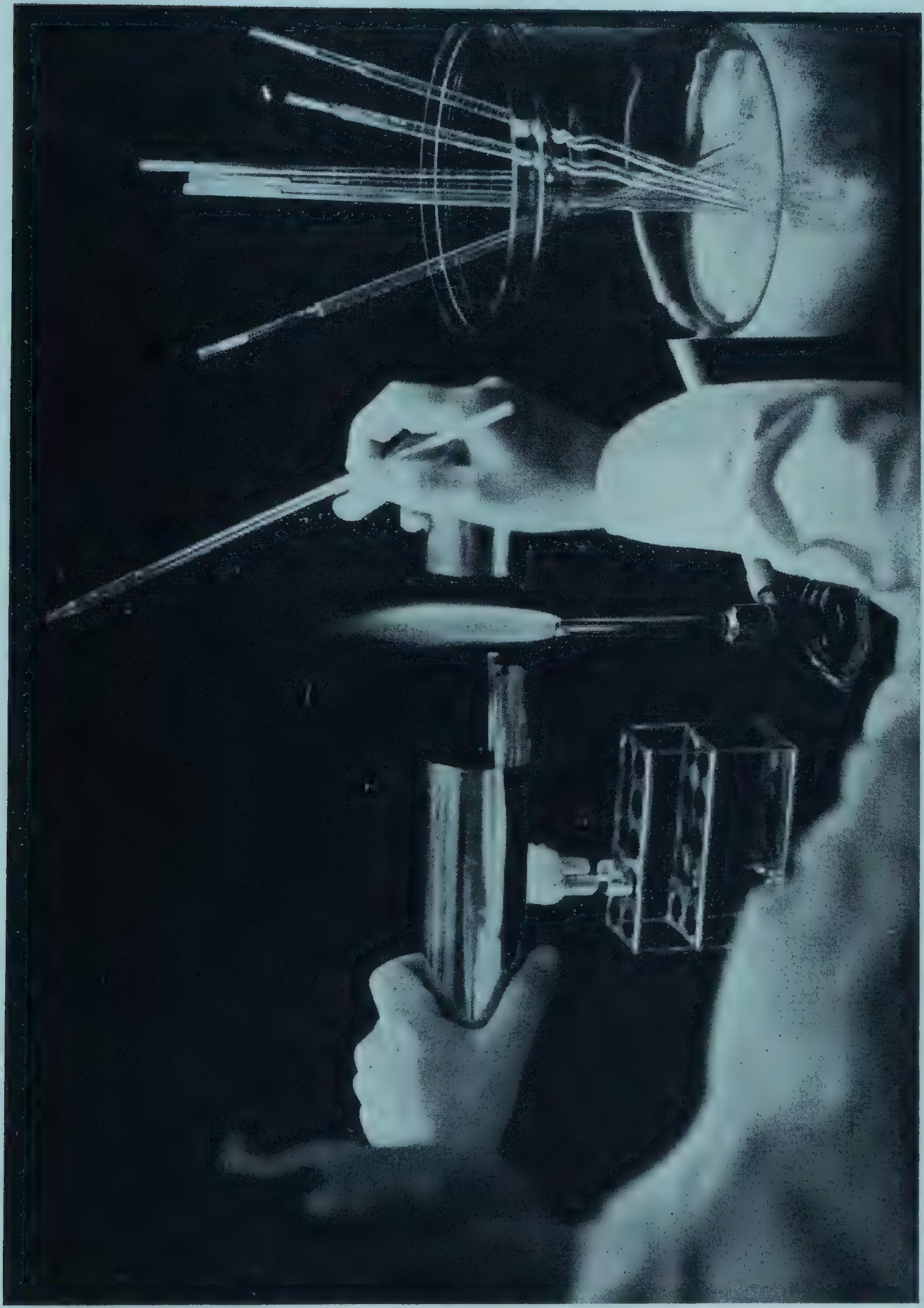

Fig. 3. Removal of Sterile Pipette from Container

Two devices are used, particularly with graduated and volumetric pipettes, to minimize the danger of introducing infective material into the mouth. The safety pipette filler (1) consists of a glass tube constricted at one end with a small vent (V) in the side, a rubber bulb (B) with holes at opposite ends fitted around the tube with V inside, and a short piece of pressure tubing placed at T for the attachment of the pipette (figure 4).

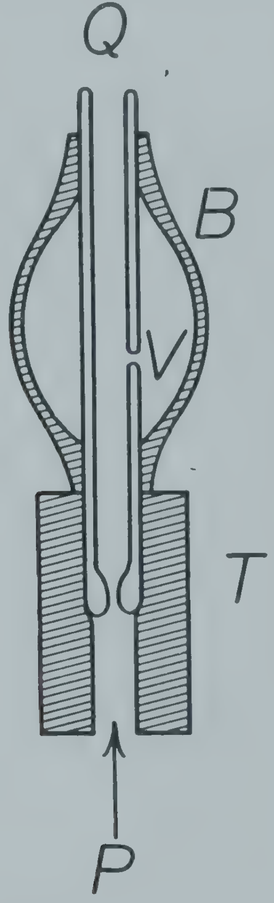

Fig. 4. Safety Pipette Filler

The filler should be made to suit working conditions, primarily by proper choice of orifice sizes at T and V. These are best characterized by E and S respectively, the time for 100 ml. of air to pass through the orifice from atmospheric pressure to a vacuum line (at less than half that pressure).

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The other device consists of a sterile glass mouthpiece attached to the pipette by a soft rubber tubing of convenient length. Pipettes are readily inserted in and removed from the rubber tubing while the mouthpiece is held firmly between the teeth, and the volume delivered is controlled by pressure on the tubing over the end of the pipette. Thus, the mouthpiece is not removed until it is discarded at the end of the operation. (See figure 5.)

INCUBATION OF CULTURES

Optimum temperatures for the growth of cultures are provided in electrically heated incubators equipped with thermal control units and with

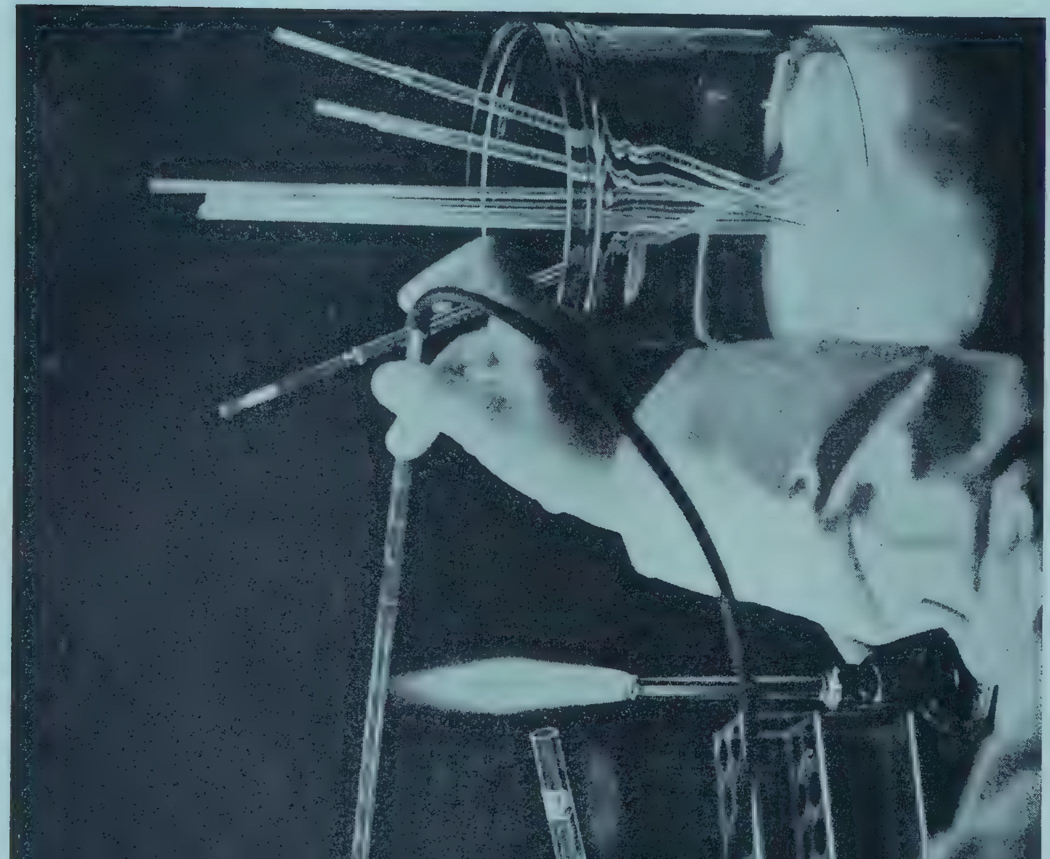

FIG. 5. PIPETTE WITH MOUTHPIECE

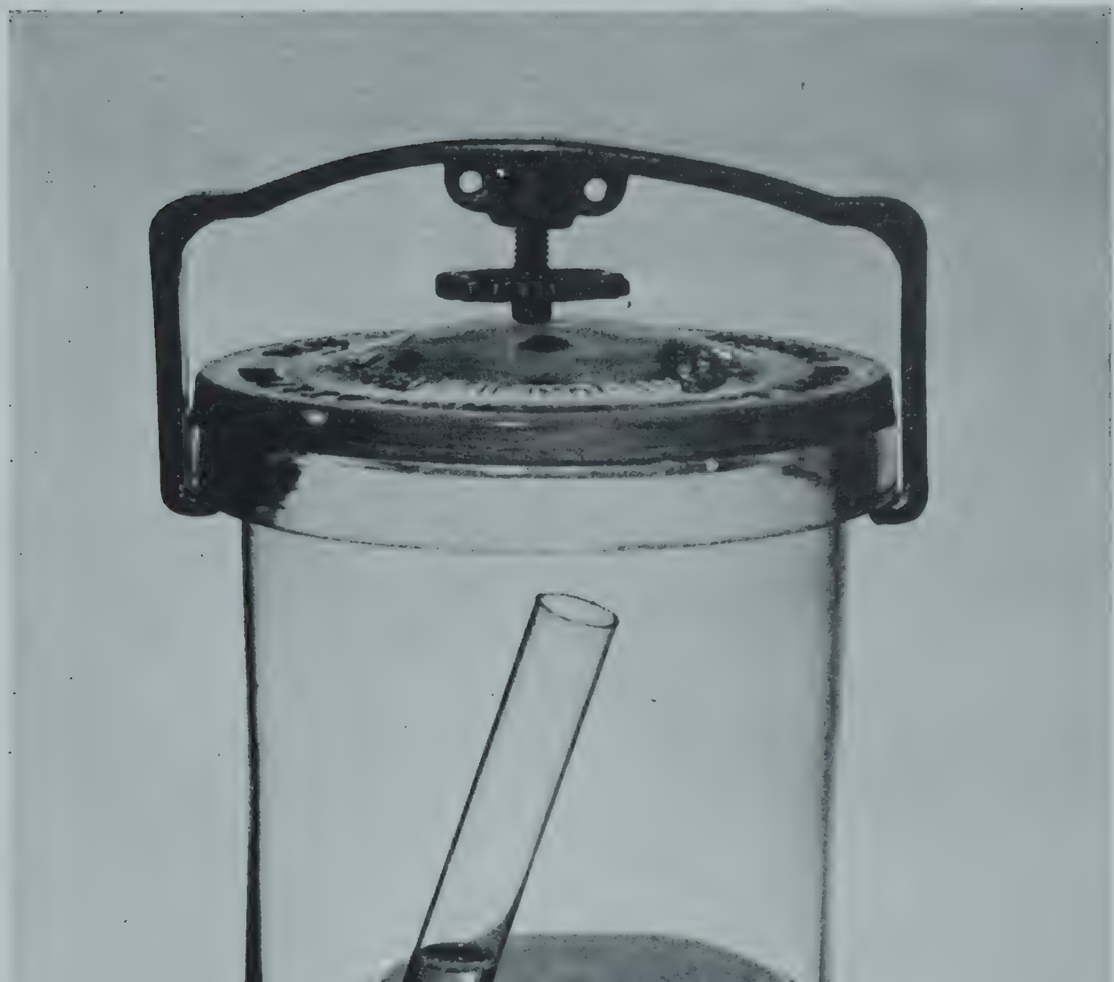

FIG. 6. CULTURES INCUBATED IN THE PRESENCE OF CARBON DIOXIDE

thermometers that record the maximum and minimum temperatures. Cultures are usually incubated for from eighteen to twenty-four hours at 35°–37°C. When other temperatures are specified a similar deviation may be assumed unless the text indicates otherwise. For some purposes cultures are grown at room temperature which is usually 20°C. but may vary from 18° to 30°C. depending upon the season of the year. The usual temperature for the temporary storage of cultures is 4°–6°C. If cultures in tubes are to be incubated or stored for several weeks, each plug is grasped with forceps and passed rapidly through a flame as it is inserted in the tube. The plug is then covered with paraffin unless the medium is otherwise protected against evaporation and contamination due to the growth of molds through the cotton plugs. Cultures in Petri plates are protected by placing the plates in tightly covered containers or by sealing each plate with bands of rubber or of gummed cellulose tape. For procedures used in prolonged storage see "Collection of Type Cultures," p. 90.

For Aerobic Growth.—Incubate inoculated media under normal oxygen tension or in the presence of from 5- to 10-per-cent carbon dioxide.

To obtain from 5- to 10-per-cent carbon dioxide, place the cultures in a container equipped with a tight-fitting cover. Generate the carbon dioxide by slowly adding 10-per-cent sulfuric acid to sodium carbonate in a tube that has been placed in the container. Approximately 1 gram of sodium carbonate and 10 ml. of acid are used for a 2½-liter jar. When the reaction begins to subside, close the container. (See figure 6.)

For Anaerobic Growth.—Immediately before subsurface inoculations are made, heat the media in a bath of boiling water for from fifteen to twenty minutes to expel absorbed oxygen; cool to 45°C.; inoculate and rotate to distribute the inoculum. To avoid the entrance of air, solidify agar or gelatin media rapidly by immersing the tubes in cold water. Liquid media and those that cannot be heated because of the nature of their ingredients may be covered with a layer of vaseline or of a mixture of paraffin and mineral oil (*F79*). Loosen these seals by heating the sides of the tubes slightly. Immediately after inoculation, place the tubes in an upright position so that the seal will harden in an even layer. A reducing agent, such as sodium thioglycollate (usually 0.05 per cent), and a suitable indicator may be incorporated in media for anaerobic growth (2, 3). Use such media soon after preparation; examine before inoculation to make sure from the color of the indicator that significant quantities of oxygen have not been absorbed.

For the cultivation of anaerobes, especially on the surface of media, incubate cultures in anaerobic jars (4, 5). By this method free oxygen is removed by oxidation of hydrogen by means of an electrically heated catalyst, usually palladium or platinized asbestos. Details of the procedures for removing the oxygen are dependent upon the construction of the lid of the jar: unless the heating element and its connections are completely enclosed in a screened chamber, the air is displaced with hydrogen before the electric current is turned on.

Place the cultures in an aluminum jar on a piece of wire gauze to prevent breakage. Put a thick even coating of a mixture of wax and vaseline (*F80* or *F80.2*) on the opposing surfaces of the lid and the jar. Prepare an indicator by mixing in a tube equal quantities of the following solutions: 0.006 *N* sodium hydroxide; 0.015-per-cent aqueous methylene blue; 6-per-cent aqueous solution of glucose (*F62A*). Boil the indicator until colorless. Immediately put it in the jar and clamp the lid in place. Open the main valve of the hydrogen tank and adjust the reducing valve until a pressure of from 1 to 2 pounds per square inch is registered on the small gauge when the rubber outlet tube is closed by pressing one finger tightly over the end.

Lid Without Screened Chamber.—Make sure that the tips and threads of the needle valves of the gas inlet and outlet on the cover are coated with wax-vaseline mixture (*F80* or *F80.2*). Connect the outlet tube of the hydrogen tank to the inlet valve on the cover of the jar, and with both valves on the jar open, flood with hydrogen for at least one minute. Close the outlet valve on the jar, then, immediately, the inlet valve, and, finally, the reducing valve on the hydrogen tank. Disconnect the outlet tube of the hydrogen tank from the jar.

Connect the heating coil in the jar with the electric current through the required outside resistance. Be sure to attach the wires to the terminal posts on the lid before plugging into the source of current. After the current has been on for ten minutes, admit hydrogen under very low pressure to insure complete removal of the oxygen and to equalize the pressure. Adjust the reducing valve on the hydrogen tank so that the flow of gas is just perceptible and the pressure barely registers on the small gauge when the outlet tube is closed by pressing one finger tightly over the end. Continue the flow of hydrogen into the jar at this low rate for from ten to fifteen minutes. During this period leave the electric current on as long as the top of the jar is cool. If the top becomes warm, turn off the current. When the run is completed, close the inlet valve on the jar tightly and disconnect the outlet tube of the hydrogen tank. Remove the plug connecting the outside resistance with the source of electric current and disconnect the wires from the terminal posts of the jar.

Lid with Screened Chamber.—Connect the outlet tube of the hydrogen tank (pressure reduced to 1–2 pounds per square inch) to the inlet on the lid and connect the heating unit to the electric current. After thirty minutes, clamp off the inlet and disconnect the hydrogen and electricity.

After completing the operation with either type of lid, close the reducing valve and then the large valve on the hydrogen tank. Release the pressure between the two valves by opening and then closing the reducing valve.

The methylene-blue indicator remains colorless as long as anaerobic conditions are maintained. A change in the color of uninoculated blood-agar medium (*F43A*) from bright red to dull purple may also serve as a control of anaerobiosis.

ISOLATION AND STUDY OF PURE CULTURES

The early procedure for the isolation of pure cultures by successive dilution in liquid media to the point of growth from a single bacterial cell was applicable only to obtaining pure cultures of the predominant species. The plate method devised by Koch and now in general use does not have this limitation; by this technic a solid medium favorable to the species sought is so inoculated that individual colonies may develop and pure cultures be obtained by transplant. By applying the principles of these methods alone or in combination with others selected in the light of the biologic differences between microorganisms, a great variety of technics for the isolation of pure cultures has been developed. Thus, selection of a procedure is dependent upon the characters of both the particular microorganisms sought and the contaminants present. Knowledge of such properties as spore formation, filterability, and pathogenicity, and of the requirements for growth—temperature, oxygen, hydrogen-ion concentration, utilization of carbohydrates, etc., may all aid in isolation. However, the basic procedure comprises, first, a microscopic examination of stained preparations, which must be kept for reference until the study is completed; then inoculation of plates of suitable solid media from which well-isolated colonies are fished; and, finally, repeated inoculation of plates with subcultures from individual colonies until the purity of the culture is established. Subsequent studies of the pure cultures are determined by the purpose of the examination. All questionable results must be compared with those of a parallel study of known strains of the species under investigation.

PLATING

Information concerning the source of the specimen, and results of the preliminary microscopic examination serve as guides in selecting the medium and conditions of incubation for plate cultures for the isolation of pure cultures. These data are essential, moreover, to the application of various procedures that supplement the plate method.

Descriptions of the following special procedures are given in those sections of the methods of the Diagnostic Laboratories that deal with individual species, or may be found in text books on bacteriology (6, 7): selective and differential media; animal inoculation; chemical treatment; agglutination; filtration; single-cell technic.

Streaked Plates.—With a loop or swab, suspend a representative portion of the culture or specimen in suitable diluent—usually broth. In parallel rows approximately 2 mm. apart, streak one loopful of the diluted material over the surface of solid medium in a Petri plate.

If only a small number of microorganisms are present, dilution may be unnecessary. An elongated loop, 5 by 12 mm., bent at an angle of approximately 30° is convenient for spreading relatively large inocula.

When contaminants may outnumber the species sought, place the inoculum on a narrow strip of the medium near and parallel to the upper left edge of the plate. With a sterile loop streak the upper half of the plate in parallel rows approximately 2 mm. apart, starting in the inoculated area and allowing the loop to pass through it several times. Turn the plate counterclockwise through an angle of 90° and streak the upper half of the plate. After a second similar rotation of the plate, again streak the upper half (8).

Poured Plates.—In a water bath, completely melt solid medium dispensed in tubes: gelatin media at from 35° to 40°C.; agar media in boiling water. Cool agar to approximately 45°C. Dilute the specimen or culture by mixing measured amounts with known volumes of diluent. Place accurately measured volumes, usually 0.5 or 1.0 ml., of the selected dilutions in Petri plates. Dry the outside of the tubes of medium with a towel; pour the contents of one tube into each Petri plate after the mouth of the tube has been carefully flamed. Take care to raise the cover of the plate only enough to admit the pipette or tube and to replace it promptly. Mix the inoculum and medium by gently tilting or rotating the plate.

Preliminary Heating Method for Sporeforming Microorganisms.—Heat part of the specimen in a water bath to destroy the vegetative forms—for example, fifteen minutes at 79°–81°C., thirty minutes at 69°–71°C. or at 59°–61°C. If the specimen is acid, neutralize with 0.05 *N* sodium hydroxide before heating. Inoculate plates of solid media with the heated material.

FISHING

If aerobic conditions are suitable or an atmosphere containing from 5- to 10-per-cent carbon dioxide is used, incubate the inoculated plates for from eighteen to twenty-four hours; if anaerobic conditions are required, for approximately forty-five hours. Reincubate the plates if necessary.

Remove the cover of the Petri plate and place it right side up on the bench; if moisture has collected on the inside of the cover, place it on a surface that has been wet with 1-per-cent cresol (*F75*). Study the colonies carefully either with a hand lens or, if the medium is translucent, under the low-power objective of a microscope. Select a well-isolated colony and transfer it with a needle to appropriate medium. With microorganisms that grow sparsely, use a loop to cut out a section of the medium that contains the colony. Use a similar technic to make fishings from poured plates. Be sure that only one colony is touched.

If the colonies are not well-isolated, inoculate additional plates either with a suspension of several representative colonies or with the original specimen, adequately diluted.

When repeated fishing is necessary to insure purity of a culture, emulsify the fished colony in approximately 5 ml. of diluent and, after flaming the loop, streak this suspension on a plate of solid medium. After incubation, select a well-isolated colony and repeat the procedure. Continue replating and fishing until the results leave no doubt of the purity of the culture. Control each step in the procedure for isolation by microscopic examination of stained preparations.

Morphologic Examinations

For morphologic examinations, a compound microscope is used, equipped with oil-immersion, high- and low-power dry objectives, and with oculars that magnify ×5 and ×7 or ×10. Before such examinations are undertaken, the worker must have a general knowledge of the principles of microscopy (9, 10) and must be trained in the procedures recommended by the manufacturer for the use and care of the instrument.

Glass slides and cover glasses must be clean and free from oily films and should be handled by grasping the edge with clean forceps. Slides are marked with the number of the culture or specimen and passed through a flame before use. Microscopic preparations are so handled and filed that contact between any two films is avoided.

When microscopic preparations are made, the culture container is always closed before the material is spread on the slide.

Dried Preparations.—To prepare films from relatively dry material for the study of the morphology of bacteria, transfer a loopful of distilled water to a slide and suspend in it a small amount of the specimen. Transfer liquid or semisolid material directly to the slide with a loop. Spread the material in a thin, even film. Unless otherwise specified in the staining procedure, dry the preparation in the air and fix it to the slide by passing slowly through the flame, film side down.

In general, films are stained by Gram's method or with aqueous alcoholic methylene blue. For these procedures, as well as for methods of demonstrating acid-fast microorganisms, capsules, flagella, etc., see "Stains and Reagents," p. 30.

In morphologic examination, observe also these general rules: Do not reuse stains and reagents. Do not use either side of blotting paper more than once. Do not touch films with forceps used for handling slides.

Moist Preparations.—For a hanging-drop preparation to study motility, place a loopful of broth culture on a cover glass. Over it, invert a hollow-ground slide that bears a ring of vaseline around the depression. (The slides are ringed by dipping a suitable applicator into melted vaseline and applying it to the slide. Convenient applicators are made by cutting a hole of slightly larger diameter than the depression in a cork or rubber stopper or by edging a tube of similar diameter with the finger from a rubber glove—the finger is inserted into the tube with the cut edge folded over the rim.) Turn the slide over quickly and press the edges of the cover glass over the vaseline to seal the chamber.

The motility of microorganisms may often be enhanced by cultivation at room temperature; in some species this character may be demonstrated only after maintenance at room temperature.

For other moist preparations, put several loopfuls of the specimen or a drop from a capillary pipette on a slide and place a cover glass over the material. If the preparation is not examined immediately, seal the edge of the cover glass with vaseline to delay evaporation.

Tests for Biochemical and Other Differential Activities

In general, each lot of medium for the examination of biochemical activities is tested before use with at least two strains known to differ in the particular property under investigation. Whenever necessary for purposes of comparison and control, known strains are included in parallel tests. Unless otherwise specified in the following procedures, cultures are examined and the results recorded at intervals, usually one, two, seven, and fourteen days, or until further observation is unnecessary. When no reaction occurs the medium must be examined for growth with special care —whenever necessary by microscopic examination or by subculture.

Hemolysis of Red Blood Cells (11).—In a tube place 0.5 ml. of a 15-hour broth (*F4*) culture or its sterile filtrate and 0.5 ml. of a 5-per-cent suspension of horse, sheep, or human red blood cells that have been washed three times in salt solution (*F64*).[1] As controls, include one tube containing 0.5 ml. of uninoculated broth and another containing 0.5 ml. of culture of a known hemolytic strain; add to each, 0.5 ml. of the cell suspension. Mix the contents of each tube thoroughly and incubate for two hours at 35°–37°C. Examine for hemolysis after one-half hour, one hour, and two hours. At the end of the 2-hour incubation period, centrifuge any tubes in which hemolysis appears incomplete or is absent, and compare with the uninoculated broth control. Record the results:

4+ complete hemolysis
3+ supernatant dark red
2+ supernatant red
+ supernatant pink
− no hemolysis

For quantitative determinations, use a 1-per-cent suspension of washed red blood cells and dilutions of culture or filtrate.

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[1] Throughout the Methods salt solution refers to 0.85-per-cent solution of sodium chloride, C. P. (*F64*).

Liquefaction of Coagulated Serum.—Inoculate and incubate coagulated serum medium (*F15*). The first evidence of digestion of the medium is frequently indicated by a small depression around the area of growth.

Liquefaction of Gelatin.—If cultures in gelatin (*F31*) are to be incubated at 20°C., completely melt the medium by heating in a water bath at 35°–40°C.; cool in a refrigerator and inoculate by stabbing the medium to the bottom of the tube with a needle; if incubated at higher temperatures, inoculate with a loop, needle, or pipette and, before examination for liquefaction, place the tubes in the refrigerator until the uninoculated medium in a control tube has solidified. Observe the test at intervals for at least three weeks unless complete liquefaction has occurred earlier.

Digestion of Meat.—Mark the level of the meat in a tube of chopped meat medium (*F6*) and inoculate. After incubation, note any changes in the height or appearance of the column of meat.

Production of Indole.—Inoculate tryptone medium (*F29A*) and incubate four days. To approximately 3 ml. of the culture add 0.5 ml. of Kovács's reagent (12) (see p. 37) and shake gently. The presence of indole is indicated by the appearance of a red color in the layer of the reagent at the top of the medium.

Nitroso-indole Reaction (Cholera Red).—Inoculate potassium nitrate tryptone medium (*F29B*) and incubate for twenty-four hours. To approximately 3 ml. of the culture add 0.3 ml. of concentrated sulfuric acid. The presence of nitroso-indole is indicated by the appearance of a distinct pink color throughout the culture.

Peptonization and Fermentation of Milk.—Inoculate milk medium containing bromocresol purple indicator (*F18B*). As a control, incubate at the same time an uninoculated tube of the medium. Compare each inoculated tube with the uninoculated control and record A or Alk when from the change in color of the indicator the acidity of the medium has increased or decreased respectively; C, coagulation; P, peptonization; −, no change.

Fermentation of Carbohydrates and Related Substances.—To test for the production of acid, inoculate media that contain the required carbohydrate, alcohol, etc., (*F5B*, *F22*, *F28*, *F39A*, or *F39B*) and, as a control, incubate at the same time an uninoculated tube of each medium. After incubation compare each inoculated tube with the uninoculated control and record the results as A when, from the change in the color of the indicator, acid has been produced; negative, −, when growth has occurred but no acid has been formed.

When serum-water media (*F28*) are used, record the results as AC when acid and coagulation are produced.

Whenever the color of the medium suggests that the indicator may have been destroyed, additional indicator should be added before recording the result, or the pH of the test culture should be determined by the spot-plate method (13).

To test for the production of gas, inoculate liquid media dispensed in containers constructed to entrap gas, such as Smith fermentation tubes or, in the case of carbohydrates not readily hydrolyzed by heat, in double-tube fermentation sets, see p. 179. After incubation, compare the level of the medium in the closed arm of the inner tube with that of an uninoculated control. Record the results as G when the formation of gas has partially displaced the medium. Record also the approximate amount of gas formed in terms of the percentage of the medium displaced.

If solid medium is used, inoculate by stabbing with a needle. After incubation, examine the inoculated medium for bubbles of gas; no bubbles due to evolution of preabsorbed air should be present in the uninoculated control.

Media for detection of the production of gas should be stored at room temperature rather than in the refrigerator in order to prevent excessive absorption of air.

Formation of Acetylmethylcarbinol (Voges-Proskauer).—Inoculate glucose-peptone solution (*F19*). After incubation for two days, to 2 ml. of the culture, add 2 ml. of 10-per-cent potassium hydroxide, mix, and examine for the presence of an eosin pink color after the test has stood for from eighteen to twenty hours at room temperature. If little or no color appears, repeat the test after incubation for four days.

Methyl Red Test.—Inoculate glucose-peptone solution (*F19*). After incubation for four days, to 2 ml. of the culture add 0.1 ml. of methyl red indicator and mix by shaking gently. Distinct red color indicates "M.R. +"; distinct yellow, "M.R. −."

Utilization of Citrate.—Inoculate the surface of citrate-agar medium containing bromothymol blue indicator (*F55*) and incubate for from eighteen to twenty hours. Examine for growth and color change. Usually *Bacterium coli* grows slightly, if at all, and does not change the color of the indicator; *Bacterium aerogenes* grows luxuriantly and changes the indicator from green to blue.

Solubility Test for Pneumococci.—To 0.1 ml. of 0.2-per-cent solution of sodium lauryl sulfate (*F73.5*) (14), or of 1-per-cent solution of sodium desoxycholate (*F73*), or of ox bile (*F59*) add 0.6 ml. of a broth (*F4* or *F5A*) culture. Pneumococci are usually lysed by either of the first two reagents within five minutes at room temperature. If ox bile is used, incubate the test at 35°–37°C. and examine after fifteen and thirty minutes and two hours; consider the strain insoluble if the fluid is not clear after two hours' incubation.

EXAMINATION OF SEROLOGIC AND PATHOGENIC ACTIVITIES

Studies of serologic and pathogenic activities are essential to confirm the results of morphologic, cultural, and biochemical examinations in the identification of microorganisms. For this purpose, tests of agglutination, precipitation, absorption of agglutinative or precipitative activities of sera, virulence, and toxigenicity are most frequently used. The basic technic for these procedures is therefore included in this section. In the identification of certain microorganisms, complement fixation, capsular swelling, toxin-antitoxin neutralization, and protection tests furnish information of practical value and are described in the sections that deal with the individual species.

For the preparation of agglutinating, precipitating, and other sera, see "Production and Standardization of Sera and Other Preparations for Diagnostic Use," p. 783.

The technic for the inoculation and autopsy of animals is outlined in the chapter, "The Use of Experimental and Test Animals," p. 73.

AGGLUTINATION

For agglutination tests, varying dilutions of the selected agglutinating sera are mixed with equal volumes of uniform suspensions of the cultures to be tested. The reactions are controlled by mixing the suspension of each culture with salt solution and with one or more dilutions (equal to smallest-fold dilutions of agglutinating serum) of serum from a normal animal of the species in which the agglutinating sera were produced; at least the largest-fold dilution of agglutinating serum is tested with a strain of the bacterial species with which the serum was prepared. The period and temperature of incubation of the test depend upon the microorganism under investigation.

Macroscopic Tube Test

Serum Dilutions.—The serum for the test should be clear; if it contains suspended precipitate, clarify by centrifugation. The dilutions vary with the potency of the sera and are usually prepared in geometric progression, e.g., 1:10, 1:20, 1:40, etc., or 1:250, 1:500, 1:1000, etc., see p. 24.

Select containers of suitable size; label each with the number and relative concentration of serum. With a pipette or burette, dispense the required amount of salt solution (*F64*). Pipette the serum into the container prepared for the highest concentration; with an unused pipette, mix thoroughly by drawing up and expelling several times and then transfer the required volume to each container for lower concentrations prepared from this dilution. Follow this procedure until all concentrations are prepared; use as few intermediate dilutions as practical without sacrifice of precision.

Bacterial Suspensions.—Saline suspensions of cultures grown for from sixteen to eighteen hours on solid media to which no fermentable carbohydrate has been added are generally employed in the test. With certain species, such as the pneumococcus, broth cultures or microorganisms grown in broth and resuspended in salt solution are used. In general, cultures of highly infective microorganisms are treated with formalin, usually 0.25 per cent, before the suspensions are prepared.

After incubation, examine each culture on solid medium for characteristic growth and for macroscopic evidence of contamination; to each one that appears satisfactory, add a small quantity of salt solution, (*F64*). Loosen the growth from the surface of the medium with a loop or with the tip of a pipette wound with a small amount of cotton. If the suspension is not homogeneous, draw it through the cotton into the pipette; discard the cotton and transfer the suspension to a clear tube that has been marked with the culture number. Repeat with each culture and dilute the suspensions with salt solution until all are of uniform turbidity as determined by comparison with a glass standard, usually No. 2.5 (p. 26) that has been dispensed in a vial approximately equal in diameter and in thickness of glass to the tubes containing the suspensions.

When the test requires only a small quantity of suspension, particularly of strains that grow heavily, dispense a sufficient volume of salt solution in a tube and add the concentrated bacterial suspension until the desired turbidity is reached.

Treat centrifuged cells from broth cultures similarly.

When sterile filters for the bacterial suspensions are required, the cotton is twisted on the tips before the pipettes are placed in cans for sterilization or small cotton filters are used. (See p. 622.)

Serum-Culture Mixtures.—Place clear, unplugged tubes (11- by 75-mm.) in racks so arranged that all tubes for a given culture are in a single row from left to right; and all tubes for a given serum dilution, in one row from front to back. With a wax pencil mark the bounds of each serum by lines across the tops of the racks, the identification of the serum between the lines on the front rack, the culture number on the tube or on the rack at the extreme left of each row. Mark the ends of the racks that come together with identifying numbers.

Begin pipetting the reagents into the tubes by placing 0.3 ml. of salt solution (*F64*) in each tube of the left row. Mix thoroughly the largest-fold dilution of normal serum and distribute 0.3 ml. in each tube in the proper row. Use the same pipette and procedure for each dilution in order until the smallest is reached. Continue with the remaining sera. Be sure to take an unused pipette for each serum and proceed from the largest- to smallest-fold dilution. To each tube in the front row add 0.3 ml. of the required bacterial suspension. Repeat with the other bacterial suspensions in the appropriate rows. When the bacterial suspensions have been distributed, flame the mouths of the tubes if living microorganisms were used. Shake the racks to ensure thorough mixing of serum and culture and place them in the incubator for the required period:

Bacterium tularense—Two hours at 35°–37°C. followed by overnight at 4°–6°C.

Bacillary incitants of enteric disease—Fifteen to twenty hours at 48°–52°C.

Brucella—Four hours at 48°–52°C. followed by overnight at 4°–6°C.

Neisseria—Twenty-four hours at 48°–52°C. for identification; two hours at 34°–36°C. and overnight in the cold room for group classification.

Pneumococcus—Two hours at 35°–37°C.

Hold the tubes in a slanted position, and before and after shaking examine for agglutination by transmitted light against a dark background. Record the results:

4+ supernatant clear; complete agglutination
3+ supernatant clear or nearly clear; definite clumping
2+ supernatant slightly turbid; definite clumping
+ supernatant turbid; small clumps definitely visible to unaided eye
± questionable clumping
− uniformly turbid suspension; no clumps

The agglutinability of microorganisms of the enteric disease group and of certain other species may often be enhanced by cultivation at room temperature for one or several days.

Macroscopic Slide Test

Prepare the selected dilutions of normal and agglutinating sera as directed for the macroscopic tube test. Suspend evenly a small amount of growth of the test culture in salt solution (*F64*)—either several loopfuls on a glass slide or a suitable volume in a small tube. On a glass slide mix one loopful of this suspension with 1 loopful of each of the diluted sera; tilt the slide back and forth for several seconds. Examine for clumping by transmitted light against a dark background; record the reaction as described for the tube test. Confirm the results by a macroscopic tube test.

With experience it is possible to so judge the amount of growth necessary for the test that preliminary suspension in salt solution may be omitted, that is, a very small amount of growth is added directly to each serum dilution with a loop or needle.

PRECIPITATION

The technical procedures and controls for precipitation tests are similar to those described for agglutination except that extracts of the bacterial cells replace the suspensions of microorganisms. The method of preparing the extract varies with the species; for example, with the pneumococcus the serologic type may be determined by tests with the clear supernatant of broth cultures; with the hemolytic streptococcus, determination of the serologic group necessitates acid extraction of the bacterial cell.

Distribute the serum, undiluted and diluted, in volumes usually of 0.3 ml. in 11- by 75-mm. tubes or of 0.1 ml. in 8- by 100-mm. tubes. Slowly add an equal volume of the extract, diluted or undiluted, or of salt solution, by allowing it to flow down the side of the tube so that two layers are formed. Incubate the test at 35°–37°C. Examine after fifteen minutes or less for the formation of a ring of precipitate at the zone of contact of the two fluids; record the results as 4+, 3+, 2+, +, ±, or −, depending upon the density of the ring. Mix the constituents by shaking the racks and reincubate for a total period of two hours. Record the final results after the test has remained at 4°–6°C. for from eighteen to twenty hours:

4+ supernatant clear; complete precipitation
3+ supernatant clear or nearly clear; definite precipitation
2+ supernatant slightly turbid; definite precipitation
+ fluid turbid; particles visible to unaided eye
± fluid slightly turbid, no particles visible
− fluid clear

When the precipitate is in the form of disks or shreds, record the results in terms of opacity, for example, opaque, semiopaque, or, transparent disk, transparent shreds.

ABSORPTION OF AGGLUTINATIVE OR PRECIPITATIVE ACTIVITIES

Absorption of agglutinative or precipitative activities of sera is used for the identification and differentiation of antigenically related microorganisms. When the identity of two closely allied strains must be established, cross-absorption (the "mirror test") (6-a) may yield practical results. By this procedure, sera of both strains are treated with suspensions of bacterial cells or with extracts of the cells of the homologous and heterologous cultures. After incubation and centrifugation, the absorbed sera are tested with both antigens. Absorption is usually directed, however, toward the removal of cross-reactivity to obtain sera that can be used to determine the presence of particular antigens in a given species or type.

For absorption of cross-reactivity from antipneumococcus, antistreptococcus, and antisalmonella sera, see p. 790, 798, and 787, respectively.

VIRULENCE

To determine virulence, measured volumes of a young actively growing culture are injected into animals. The species of animal, usually rabbit, guinea pig, rat, or mouse, and the method of inoculation depend upon the microorganism under investigation. The animals are observed closely for evidence of infection and an autopsy is performed as soon after death as possible. Bacteriologic and, whenever necessary, histopathologic examinations are made of any lesions that may be present.

Dilute the growth from an actively growing culture in broth so that the desired dose is contained in a volume suitable for injection. Record the weight and, if required, the temperature of the animal, and administer the test material by injection into the subcutaneous tissue, peritoneum, or vein; or by instillation into the nose or stomach. If several dilutions of each culture are to be tested in different animals, inoculate an animal with the largest-fold dilution first and progress in order to the smallest, rinsing the syringe with each dilution. Be sure to use a sterile syringe for each strain. When it is desirable to determine the approximate number of microorganisms injected, inoculate poured plates of suitable medium in duplicate with accurately measured volumes of the culture suspension. Incubate the plates at least forty-eight hours and count the colonies that develop.

To obtain satisfactory plates for counting, it may be necessary to make poured plates with several large-fold dilutions which differ by 10- or 100-fold.

Examine the inoculated animals daily. When death occurs, take cultures and prepare films for microscopic examinations from the site of inoculation, if practicable, from the heart's blood, and from any lesions that are found. Remove tissue for examination. See p. 85.

TOXIGENICITY

Toxigenic activity is measured by the reaction induced in animals by sterile filtrates of bacterial cultures. The selection of the test animal and method of injection depend upon the bacterial species under investigation. For purposes of control, a heated portion of the test filtrate, the heated and unheated toxin of a representative strain of the same species, and uninoculated broth similar to that in which the cultures were grown are also injected.

Prepare dilutions of the sterile filtrate and of the control toxin in salt solution (*F64*). After thorough mixing, remove portions of the smallest-fold dilution used in the test and heat sufficiently to destroy the control toxin. Prepare a dilution of uninoculated broth equal to the smallest-fold dilution. Record the necessary data concerning the weight, etc., of the test animals. For the inoculation, use different syringes for filtrate, heated filtrate, and uninoculated broth. If several dilutions of the filtrates are injected with one syringe, be sure to proceed from the largest- to smallest-fold dilutions, rinsing the syringe with each.

Compare the reactions induced by the test filtrate, the known toxin and the controls. Consider the test unsatisfactory if definite reactions are induced by the broth and heated filtrate controls. Examine thoroughly any animals that die, and remove material for bacteriologic and histopathologic examination whenever indicated.

A similar technic is used for testing killed bacterial suspensions for toxicity.

For details of tests to determine the toxigenicity of particular microorganisms, see sections on individual species.

Preparation of Dilutions

The word dilution has been used widely with the following meanings:

I. The dilution factor as explained in the text
II. The process of diluting
III. The mixture resulting from Process II
IV. The relative concentration.

Obviously meanings I and IV are quantitative meanings and are reciprocal; thus I increases as IV decreases. The two uses are confusing and IV is objectionable although it has been used extensively. Throughout the volume whenever a statement is made about higher or lower dilutions it is to be interpreted in the sense of I, that is, greater dilutions mean lower concentrations. Where ratios are given, as for example, "Use the following serum dilutions: 1:200, 1:500, 1:750 . . ." it is to be understood that the ratios are relative concentrations. The third meaning of the word dilution is intended in such instances. Forms such as "the dilution equals 1:200," or "the dilution is 1:200" should be avoided as coming under meaning IV. Statements such as "dilutions of 1:200" are admissible under meaning III but are best avoided also, as the word dilution may be misinterpreted to mean IV.

In the preparation of dilutions of sera, toxins, or cultures, carefully measured volumes of the reagent and diluent are thoroughly mixed in containers of suitable size. Procedures are based on the following relations:

Fundamental relations: It is convenient to express in the form of a fraction the relative concentration, C, of a given reagent employed in a preparation. Suppose that a diluent is admixed with v ml. of the reagent until the volume of the mixture is V ml. Then the relative concentration, C, of reagent in the mixture is v/V by definition.

Let δ ml. be the amount of diluent thus added. Then

$$V = v + \delta, \tag{1}$$

to a good approximation in cases to be considered; although in others, as when alcohol or sulfuric acid is diluted with water, V may be considerably less than $v + \delta$. In the following development (1) will be considered as true.

For convenience let D be defined by the relation

$$C = 1/D;\ \text{i.e., } D = 1/C. \tag{2}$$

D may be called the dilution factor, and the process may be called a D-fold dilution. By definition $C = v/V$, whence $V = vD$, and $V - v = vD - v = v(D - 1)$. Accordingly (1) gives

$$\delta = v(D - 1). \tag{3}$$

This furnishes a convenient means of estimating the amount (δ ml.) of diluent to be added to a given amount (v ml.) of reagent to make the concentration in the mixture as required ($C = 1/D$), i.e., to effect a D-fold dilution. Thus, to make a one in ten dilution, $C = 0.1$, $D = 10$, and $\delta = 9v$. To each milliliter of reagent 9 ml. of diluent is added. The dilution factor here is ten, and the process may be called a ten-fold dilution. To make a two-and-one-half-fold dilution ($D = 2.5$), then $\delta = 1.5v$; and to each milliliter of reagent 1.5 ml. of diluent is added. When D is large, it may be convenient to make successive dilutions where the product of all dilution factors is made equal to D. Then the final mixture is a D-fold dilution of the original reagent.

Simple dilution: Suppose it is required to find how much to dilute a quantity (V_0) of a preparation of known strength or titer (C_0) to yield a preparation of strength (C). Then the required total volume V is given by

$$(4) \qquad V = \frac{C_0 V_0}{C}\text{; i.e.,}$$

$$\text{required total volume} = \frac{\text{(present strength) (present volume)}}{\text{required strength}}.$$

The amount of diluent to add is the difference between these volumes, and may be estimated directly by

$$(5) \qquad V - V_0 = \frac{V_0 (C_0 - C)}{C}\text{; i.e.,}$$

$$\text{volume to add} = \frac{\text{(present volume) (present strength} - \text{desired strength)}}{\text{desired strength}}.$$

Preparation of a required volume of specified strength: Suppose it is required to make a specified volume (V) of preparation of strength (C) from a stock preparation of strength (C_0). The required volume (V_0) of the stock to be diluted is found at once from relation (4) by

$$(6) \qquad V_0 = \frac{C \cdot V}{C_0}\text{; i.e.,}$$

$$\text{volume of stock to be used} = \frac{\text{(required strength) (required volume)}}{\text{stock strength}}.$$

This amount of stock is diluted to the required volume (or diluent is added in volume equal to the difference). If the calculated amount of stock is too small to measure with satisfactory precision (e.g., less than 0.5 ml.) it may be preferable to make an intermediate N-fold dilution and use N times as much of this intermediate preparation to dilute to the required volume. Various combinations and sequences of dilution are possible, and several intermediate dilutions may be employed provided the product of all dilution factors in the sequence is C_0/C; e.g., a thousand-fold dilution may be made by factoring $1000 = 10(100) = 20(50) = 40(25) = 10(10)(10)$, etc., and using any set of factors in any order in successive dilutions. Bacterial suspensions may be prepared in accordance with these directions by expressing strength appropriately (e.g., as microorganisms per ml.).

Addition of preservative: Suppose a preservative in stock solution of strength (C_0) is to be added to a given volume (W) of a preparation (e.g., vaccine) to give a final strength (C) of preservative. The volume of diluent (W) is given, and it is required to find the volume (V_0) of stock solution needed. Then $W = V - V_0$ in relation (5) where V is the final total volume; and

$$V_0 = \frac{C \cdot W}{C_0 - C}\text{; i.e.,} \tag{7}$$

the volume of stock preservative solution to be added =

$$\frac{\text{(required preservative concentration) (volume of present preparation)}}{\text{preservative concentration in stock solution} - \text{required concentration}}.$$

Preparation of reaction mixtures: A convenient table is appended, which illustrates and suggests appropriate methods of preparing simple or successive dilutions to study reactions with variously specified reagent concentrations in the reaction mixture. (See table 1.)

Preparation of Turbidity Standards (15–17)

Stock Suspensions of Pyrex Glass: Break discarded pyrex glassware into ¾-inch or smaller chips, place a layer 2½ inches deep in a Pyrex bottle, 6¾ inches in diameter and fitted with a pyrex glass stopper. Clean the chips and bottle with sulfuric-chromic acid cleaning mixture (*F78A*) and rinse free from acid with distilled water. Just cover the chips with distilled water and shake for six hours in a machine operating at 130 cycles per minute with a 2-inch stroke, which should provide for violent agitation of the chips. Discard the water and repeat the shaking process with a fresh portion. Discard, and again cover the chips with distilled water and shake for the third time until the suspension approximates the opacity of skim milk. The total time required will vary from twenty-four to one hundred and twenty hours (not necessarily consecutive), depending upon whether freshly broken chips are used.

Transfer the suspension to 1-liter cylinders, cover, and let stand for forty-eight hours to allow the coarser particles (more than 2 microns) to settle. Decant, allow the supernatant to stand for an additional twenty-four hours, and decant again. Determine the turbidity of the decanted suspension in a Klett-Summerson (KS) photoelectric colorimeter. Use test tubes and blue filter No. 42. Dilute with distilled water if necessary to bring the KS reading to 590. If too dilute, return the suspension to the original bottle containing the glass chips, shake further in the machine, and repeat the settling and decanting procedure. Dilute to a standard turbidity equivalent to a KS reading of 590 and determine the dry solids content. If less than 0.20 per cent, return the suspension to the cylinders used for the second decantation, mix, and let settle for a shorter period to secure a higher proportion of large particles; if more than 0.25 per cent, use a longer settling period.

Prepare standards by dilution and calibration with a Klett-Summerson photoelectric colorimeter as indicated in table 2. The diluent is usually distilled water.

Dispense the suspensions in round-bottomed vials, 15- or 19-mm. outside diameter, marked at the initial liquid level, and equipped with molded screw caps. Examine the suspensions with the Klett-Summerson photoelectric colorimeter at intervals of six months, or whenever the liquid level becomes lower than the graduation mark or the vial, and adjust the concentration if necessary. Shake the standards immediately before use to ensure a uniform suspension.

Make standards resembling cultures in beef-extract broth (*F14A*) or bile-peptone medium (*F23*) by combining the amount of stock Pyrex suspension given in table 2 with 33⅓ ml. of the required-color solution and diluting to 100 ml. with distilled water.

TABLE 1

Scheme for dilution of a preparation A with salt solution to form a more dilute preparation, B, for admixture with a prepared second reagent in equal volume

RELATIVE CONCENTRATION IN A	VOLUME OF A USED	VOLUME OF DILUENT USED	RESULTANT RELATIVE CONCENTRATION IN B	FINAL RELATIVE CONCENTRATION IN REACTION MIXTURE
	ml.	*ml.*		
1	0.5	4.5	1/10	
1/10	1.0	4.0	1/50	1/100
1/10	1.0	9.0	1/100	1/200
1	0.1	9.9	1/100	1/200
1/100	2.0	1.0	1/150	1/300
1/100	1.0	1.0	1/200	1/400
1/100	1.0	1.5	1/250	1/500
1/100	1.0	2.0	1/300	1/600
1/100	1.0	2.5	1/350	1/700
1/100	1.0	3.0	1/400	1/800
1/100	1.0	3.5	1/450	1/900
1/100	1.0	4.0	1/500	1/1000
1/100	1.0	4.5	1/550	1/1100
1/100	1.0	5.0	1/600	1/1200
1/100	1.0	5.5	1/650	1/1300
1/100	1.0	6.0	1/700	1/1400
1/100	1.0	6.5	1/750	1/1500
1/100	1.0	7.0	1/800	1/1600
1/100	1.0	7.5	1/850	1/1700
1/100	1.0	8.0	1/900	1/1800
1/100	1.0	8.5	1/950	1/1900
1/100	1.0	9.0	1/1000	1/2000
1/100	0.5	7.0	1/1500	1/3000
1/100	0.5	9.5	1/2000	1/4000
1/100	0.2	4.8	1/2500	1/5000
1/100	0.2	5.8	1/3000	1/6000
1/100	0.2	6.8	1/3500	1/7000
1/100	0.2	7.8	1/4000	1/8000
1/100	0.2	8.8	1/4500	1/9000
1/100	0.2	9.8	1/5000	1/10000
1/100	0.2	10.8	1/5500	1/11000
1/100	0.2	11.8	1/6000	1/12000

Note: 1 = 1/1 as relative concentration means undiluted first reagent.

Use a dilution of one volume of color solution with 2 volumes of distilled water for the zero setting of the colorimeter. Since the colored standards are acid, make sure that the caps of the round-bottomed vials in which the standards are dispensed are acid-resistant.

"Beef-extract Broth" Color Solution: Dissolve 1.245 grams of potassium chloroplatinate, K_2PtCl_6, and 1 gram of cobalt chloride $CoCl_2 \cdot 6H_2O$, in distilled water. Add 100 ml. of concentrated hydrochloric acid and dilute to 500 ml.

"Peptone-bile Medium" Color Solution: Dissolve 2 grams of potassium chloroplatinate, K_2PtCl_6, and 3.75 grams of copper sulfate, $CuSO_4 \cdot 5H_2O$, in distilled water. Add 100 ml. of concentrated hydrochloric acid and dilute to 500 ml.

TABLE 2

Preparation of turbidity standards

TURBIDITY STANDARD	APPROXIMATE CONCENTRATION OF STOCK SUSPENSION	TURBIDITY AS INDICATED BY KLETT-SUMMERSON COLORIMETER
No.	%	*Scale reading (KS)*
0.5	3.32	40
1	8.34	100
2.5	24.2	250
3	30.0	290
4	39.6	360
5	52.2	420
6	60.5	460

Antiseptic Mouthwash (18)

Sodium chloride	8.0 grams
Sodium bicarbonate	2.8 grams
Water (distilled)	250.0 ml.
Glycerol	125.0 ml.
Alcohol, 95-per-cent	500.0 ml.
Thymol	0.28 gram
Menthol	0.28 gram
Oil of wintergreen	0.7 ml.
Oil of cinnamon	0.5 ml.
Oil of eucalyptus	1.2 ml.
Tincture of cudbear	24.0 ml.

Dissolve the thymol, menthol, oil of wintergreen, oil of cinnamon, and oil of eucalyptus in the alcohol. Add the remainder in the following order and mix after each addition: Glycerol; sodium chloride dissolved in 125 ml. of water; sodium bicarbonate dissolved in 125 ml. of water; tincture of cudbear.

All the reagents are U.S.P. grade. Formula is slightly modified from original.

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CHAPTER 2

STAINS AND REAGENTS. METHODS OF APPLICATION

Only those stains and reagents are included in this chapter that are in more or less general use or may be required in more than one procedure. Formulas are given in "Histologic Procedures," p. 546; under "Formulas for Media and Auxiliary Preparations," p. 183; and in other chapters when the stains or reagents are limited to a specific use.

Stains certified by the Commission on Standardization of Biological Stains (1) and chemicals of C.P. grade or the equivalent are employed. All stains are prepared from powdered dyes, except Giemsa's which may be purchased in solution. All new lots of stains should be employed only after they have been carefully tested and, if necessary, standardized.

Unless otherwise specified, ethyl alcohol; distilled water, soft filter paper, and anhydrous salts are used; solutions are aqueous; the methyl alcohol is absolute, neutral, and acetone-free. Reagent methyl alcohol supplied by Merck has been found satisfactory.

Absolute alcohol is the U.S.P. anhydrous product (99 per cent or more).

Ninety-five-per-cent alcohol is the U.S.P. product. It contains approximately 92.3 per cent C_2H_5OH by weight. Concentrations of ethyl alcohol in per cent implies volume per cent; i.e., the number of milliliters of 100-per-cent ethyl alcohol per 100 ml. of solution.

Ammonium hydroxide, concentrated, means a solution containing about the equivalent of 26–28 per cent NH_3 by weight.

Hydrochloric acid, concentrated, is a solution about 37 per cent HCl by weight.

Nitric acid, concentrated, is a solution about 70 per cent HNO_3 by weight.

Acetic acid crystal violet solution (for leucocyte count)

Crystal violet	0.2 gram
Acetic acid, glacial	5 ml.
Water to make	100 ml.

Filter before use.

Procedure.—See "Examination of Blood," p. 505.

Albert's differential stain (2, 3) (for Corynebacteria)

Toluidine blue	0.15 gram
Methyl green	0.2 gram
Acetic acid, glacial	1 ml.

Alcohol, 95-per-cent	2 ml.
Water	100 ml.

Let stand for twenty-four hours and filter.

Procedure.—Fix the slide preparations in a flame. Apply the stain for three to five minutes. Wash in tap water. Apply Lugol's iodine solution (p. 37) for one minute. Wash in tap water; blot dry.

Benedict's solution (4, 5) (for sugar)

For qualitative tests

Copper sulfate crystals $CuSO_4 \cdot 5H_2O$	17.3	grams
Sodium citrate $Na_3C_6H_5O_7 \cdot 2H_2O$	173	grams
Sodium carbonate, Na_2CO_3	100	grams
Water to make	1000	ml.

With the aid of heat, dissolve the sodium citrate and carbonate in about 600 ml. of water. Filter into a glass graduate and make up to 850 ml. with water. Dissolve the copper sulfate in sufficient water to give a final volume of 150 ml. Pour the carbonate citrate solution into a large beaker and add the copper sulfate solution slowly with constant stirring. After being mixed, the solution is ready for use.

For quantitative tests

Copper sulfate crystals, $CuSO_4 \cdot 5H_2O$	18.0	grams
Sodium carbonate, Na_2CO_3	100	grams
Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$	200	grams
Potassium thiocyanate, KCNS	125	grams
Potassium ferrocyanide, $K_4Fe(CN)_6 \cdot 3H_2O$, 5-per-cent solution	5	ml.
Water to make	1000	ml.

With the aid of heat, dissolve the carbonate, citrate, and thiocyanate in about 700 ml. of water, and filter. Weigh the copper sulfate on an analytical balance, dissolve in 100 ml. of water, and pour slowly into the other fluid, stirring constantly. Add the ferrocyanide solution, cool, and add sufficient water to make a final volume of 1000 ml. This solution keeps well and is of such strength that 25 ml. are reduced by 0.05 gram of glucose.

For purposes of control, test the reagent with a solution of glucose of known concentration.

Procedure.—See "Examination of Urine," p. 514.

Benzidine reagent (for blood)

Benzidine	1 gram
Acetic acid, glacial	10 ml.
Hydrogen peroxide, 3-per-cent	11 ml.

Test the hydrogen peroxide before use by adding to 2 ml. a few drops of a solution of potassium dichromate in sulfuric acid. If the peroxide is still active, a blue color will develop. Just before use, combine the benzidine and glacial acetic acid and add an equal volume of hydrogen peroxide.

Procedure.—Make a slide preparation from the material and pour the reagent over it. If blood is present, especially in large amounts, a blue color appears almost instantaneously.

Cresyl blue, brilliant (*6*) (*for staining reticulocytes*)

Cresyl blue, brilliant	0.3 gram
Alcohol, 95-per-cent	100 ml.

Filter before use.

Procedure.—See "Examination of Blood," p. 506.

D'Antoni's iodine solution (*7*) (*for cysts*)

Potassium iodide, standard 10-per-cent solution	10 ml.
Water to make	100 ml.
Iodine	1.5 grams

Powder the iodine and add to the 1-per-cent potassium iodide solution. Allow to stand for four days, then filter. Store in a tightly stoppered bottle.

Potassium iodide, standard 10-per-cent solution

Potassium iodide	100 grams
Water to make	1000 ml.

Place the potassium iodide in a clean 1000-ml. volumetric flask and add the distilled water.

Weigh a clean absolutely dry certified 25-ml. volumetric flask to the fourth decimal point. Fill with the potassium iodide solution to the 25-ml. mark. Weigh it again to the fourth decimal point. Subtract the weight of the empty flask from that of the full one. This gives the weight of 25 ml. of the potassium iodide solution, which, theoretically should be 26.925 grams, if it contains 10-per-cent potassium iodide. However, since potassium iodide is deliquescent, the actual weight (W) of the solution will be found to be less than the theoretical. The amount of potassium iodide to add may be estimated as $100\left(\frac{W - 26.925}{W - 25}\right)$ grams per liter.

Procedure.—See "Examination for Protozoa," p. 482.

Ehrlich's diazo reagent (*8, 9*) (*for bilirubin*)

Solution I

Sulfanilic acid	1 gram
Hydrochloric acid (sp. gr. 1.19)	15 ml.
Water	1000 ml.

Solution II

Sodium nitrate	0.5 gram
Water	100 ml.

Mix 25 ml. of solution I with 0.75 ml. of solution II. Prepare fresh before use.

Procedure.—See "Examination of Blood," p. 509.

Esbach's reagent (10) (for quantitative determination of albumin)

Picric acid	1 gram
Citric acid	2 grams
Water to make	100 ml.

Heat in water bath without boiling.

Procedure.—See "Examination of Urine," p. 514.

Field's stain (11) (for malaria parasites)

Solution I

Methylene blue	0.8 gram
Azure B	0.5 gram
Disodium phosphate, Na_2HPO_4	5 grams
Monopotassium phosphate, KH_2PO_4	6.25 grams
Water	500 ml.

Dissolve the phosphate salts in the water. Dissolve the azure B by grinding in a mortar with a small amount of the phosphate solution. Add the stain to the phosphate solution.

Solution II

Eosin Y, water soluble	1 gram
Disodium phosphate, Na_2HPO_4	5 grams
Monopotassium phosphate, KH_2PO_4	6.25 grams
Water	500 ml.

Dissolve the phosphate salts in the water and add the stain.

Allow solutions I and II to stand for twenty-four hours. Filter. Store in covered jars, maintaining the depth of the stains to about 3 inches. Standardize before use.

If a scum appears on the surface or dye precipitates on the stained films, refilter. The same solutions may be used continuously for several weeks. Renew the eosin solution, however, when it becomes greenish.

Procedure.—Dip the film into solution I for one to five seconds, depending upon the stain. Remove and immediately rinse by gently waving in clean water for a few seconds until the stain ceases to flow from the film and the glass slide is free from stain. Dip into solution II for one to five seconds; rinse by gently waving for two or three seconds in clean water. Place in a vertical position to dry.

Flagella stain (Gray) (12)

Solution I

Potassium aluminum sulfate (potash alum), $Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$, saturated solution (12 grams per 100 ml. water)	5 ml.
Tannic acid, 20-per-cent solution	2 ml.
(if a large quantity is prepared, add a few drops of chloroform)	
Mercuric chloride, saturated solution (6 grams per 100 ml. water)	2 ml.

Solution II

Basic fuchsin	10 grams
Alcohol, 95-per-cent	100 ml.

On the day on which the stain is to be used and preferably about two hours previously, combine 9 ml. of solution I and 0.4 ml. of solution II and mix by rapid rotation of the test tube. This preparation, which acts as a mordant, deteriorates rapidly.

Procedure.—Clean slides carefully. Leave in cleaning solution (*F78A* or *F78B*) for at least fifteen minutes. Wash thoroughly in running tap water. Cover with 95-per-cent alcohol, each 25 ml. of which contains from 0.5–1 ml. of ammonium hydroxide, for fifteen minutes. Dry with lens paper or soft fat-free tissue. Do not touch the surfaces of the clean slides with the fingers. Prepare in sterile water, a light suspension of a 24- to 72-hour agar-slant culture; let stand at room temperature for from fifteen to twenty minutes. Place several very small loopfuls of the suspension on a slide and dry in the air. Flood the slides with the mordant and allow to act at room temperature for ten minutes. Wash with a gentle stream of water until no more precipitate is removed. Apply a few drops of Ziehl-Neelsen's carbolfuchsin (p. 43) for from five to ten minutes. Wash with tap water.

Both the soma and flagella are stained pink.

Fontana's stain (13–16) (for spirochetes)

Ruge's fluid

Acetic acid, glacial	1 ml.
Formalin, neutral	2 ml.
Water	100 ml.

Tannic acid phenol solution

Tannic acid	2.5 grams
Phenol, 5-per-cent solution	10 ml.
Water to make	40 ml.

Ammoniacal silver nitrate solution

Silver nitrate	1 gram
Water	50 ml.
Ammonium hydroxide, concentrated (about the equivalent of 26–28 per cent NH_3 by weight)	0.5–1 ml.

When the salt is dissolved, add ammonium hydroxide drop by drop. A brownish precipitate will be formed. Continue to add ammonium hydroxide cautiously until the brown precipitate disappears and a faint milky appearance remains. If excess is added, the solution clears and may be readjusted by the cautious addition of more silver nitrate (10-per-cent solution). This stain is satisfactory for use for only a few hours after preparation.

Procedure.—The films should be thinly spread on clean slides. Do not fix in a flame. Apply Ruge's fluid for one minute, and wash in water. Cover the slides with the tannic acid phenol solution and steam for thirty seconds. Quickly wash in water and blot dry. Cover with ammoniacal silver nitrate solution and steam for thirty seconds. Wash in water; blot dry.

Spirochetes are stained from brown to black.

Giemsa's stain (17, 18)

Giemsa's stain, powder, commercial preparation	0.5 gram
Glycerol, neutral	33 ml.
Alcohol, methyl	33 ml.

Dissolve the powder in the glycerol by heating for one and one-half hours at 55°–60°C. Add the methyl alcohol.

or

Giemsa's stain, solution, commercial preparation

Buffer solution, pH 7.0

Disodium phosphate, $M/15$, Na_2HPO_4	61.1 ml.
Monosodium or monopotassium phosphate, $M/15$, $NaH_2PO_4 \cdot H_2O$ or KH_2PO_4	38.9 ml.
Water	900 ml.

Check the pH and adjust if necessary. Store in pyrex glass in a refrigerator and use for no longer than one month after preparation.

Procedure.—For thick films: Do not fix. Place in Giemsa's stain freshly diluted 1:50 in pH 7.0 buffer solution for forty-five minutes. Place in buffer solution for three to five minutes. Dry in the air; never blot.

For thin films: Fix in methyl alcohol for two to three minutes. Place in Giemsa's stain freshly diluted 1:50 in pH 7.0 buffer solution for forty-five minutes. Dip in buffer solution once or twice. Dry in the air; never blot.

Goodpasture's stain (19) (for Negri bodies)

Alcohol, 20-per-cent	100 ml.
Phenol	1 gram
Aniline	1 ml.
Basic fuchsin	0.5 gram

Since the mixture deteriorates, it should be freshly prepared at 2-week intervals.

Procedure.—See examination for evidence of rabies, p. 349.

Gowers's solution (54, 55) (for erythrocyte count)

Sodium sulfate, Na_2SO_4	7.5 grams
Acetic acid, glacial	20 ml.
Water	120 ml.

Procedure.—See "Examination of Blood," p. 504.

Gram's method (20) (for differentiation of bacteria)

Aniline crystal violet solution (21)

Aniline	40 ml.
Water	1000 ml.
Crystal violet, saturated alcoholic solution (about 10 grams per 100 ml. 95-per-cent alcohol)	114 ml.

Shake the aniline and water in a closed container until thoroughly mixed. Filter through 4 sheets of filter paper moistened with water. Add crystal violet solution to this filtrate. Prepare a new supply at least once a week and filter daily before use.

Gram's iodine solution

Iodine	1 gram
Potassium iodide	2 grams
Water	300 ml.

Grind the iodine and the potassium iodide in a mortar until thoroughly mixed. Add water slowly.

Safranine, 0.1-per-cent solution

Safranine, saturated alcoholic solution (about 2.5 grams per 100 ml. 95-per-cent alcohol)	10 ml.
Water	90 ml.

Procedure.—Apply aniline crystal violet for two minutes. Blot dry. Stain with Gram's iodine for one minute; blot dry. Cover with 95-per-cent alcohol until decolorized. Wash in running tap water. Counterstain with 0.1-per-cent solution of safranine for thirty seconds. Wash in running tap water. Dry by blotting or draining.

To control this stain, place at the end of each slide a film preparation of *Bacterium coli* and one of *Staphylococcus* from 18- to 24-hour cultures. Gram-positive microorganisms are stained a deep purplish blue and Gram-negative, pink. Consider the staining procedure satisfactory only when the *Bact. coli* is Gram-negative and the *Staphylococcus* Gram positive, or, when polymorphonuclear leucocytes are present, the nuclei retain some of the crystal violet.

Hayem's solution (52, 53) (for erythrocyte count)

Mercuric chloride	0.5	gram
Sodium sulfate, Na_2SO_4	5	grams
Sodium chloride	1	gram
Water to make	200	ml.

Gowers's Solution (54, 55) is also recommended.

Procedure.—See "Examination of Blood," p. 504.

Heidenhain's iron hematoxylin stain (22) (for protozoa)

Mordant: Iron alum solution

Ferric ammonium sulfate violet crystals, $FeNH_4(SO_4)_2 \cdot 12H_2O$	4 grams
Water	100 ml.

Hematoxylin

Hematoxylin	1 gram
Alcohol, absolute	10 ml.
Water	90 ml.
Thymol	1 small crystal

Stopper with a cotton plug and ripen for about one month. For use, mix equal quantities of the ripened solution and water.

Procedure.—See "Examination for Protozoa," p. 482.

Hiss's stain (23) (for capsules)

Crystal violet, 0.5-per-cent solution

Crystal violet, saturated alcoholic solution (about 10 grams per 100 ml. 95-per-cent alcohol)	5 ml.
Water	95 ml.

Mix and filter.

Copper sulfate, 25-per-cent solution

Copper sulfate crystals, $CuSO_4 \cdot 5H_2O$	20 grams
Water	80 ml.

Dissolve the copper sulfate in the water with the aid of heat and filter.

Procedure.—Do not fix the slide preparations in a flame. Apply 0.5-per-cent crystal violet solution and heat until steaming. Remove the stain by washing with 25-per-cent solution of copper sulfate. Blot dry.

The following modification by *Wadsworth* (24) has been found useful with material from the peritoneum of the mouse and with cultures to which serum has been added: after the preparation is dry, cover with formalin. Let stand for a few seconds, drain, blot dry. Stain by Hiss's method.

The bacterial cells are stained dark purple and the capsules, light blue.

Kovács's reagent (25) (for indole)

P-dimethylaminobenzaldehyde, $(CH_3)_2NC_6H_4CHO$	5 grams
Alcohol, amyl or butyl	75 ml.
Hydrochloric acid (sp. gr. 1.19)	25 ml.

Heat the alcohol and aldehyde in a water bath at from 50° to 60°C. until dissolved, or overnight in a 56°C. incubator. Cool and add the hydrochloric acid slowly. Store in the dark in a glass-stoppered dark bottle.

Procedure.—See "General Bacteriologic Technic," p. 17.

Lugol's iodine solution

Iodine	2 grams
Potassium iodide	4 grams
Water	100 ml.

Grind the iodine and the potassium iodide in a mortar until thoroughly mixed. Add water slowly. A fresh solution should be made weekly.

Methylene blue alkaline stain (Loeffler)

Methylene blue, saturated alcoholic solution (about 1.5 grams per 100 ml. 95-per-cent alcohol)	30 ml.
Potassium hydroxide, 0.01-per-cent solution	100 ml.

Mix and filter.

Procedure.—Allow the stain to act for at least one minute. Wash in running tap water. Dry by blotting or draining.

Methylene blue stain, aqueous alcoholic solution (1a)

Methylene blue	0.3 gram
Alcohol, 95-per-cent	30 ml.
Water	100 ml.

Dissolve the dye in the alcohol, add water, and filter.

Procedure.—Allow the stain to act for at least one minute. Wash in running tap water. Dry by blotting or draining.

See "Examination for Corynebacterium," p. 287.

Methylene blue safranine stain (26, 27) (for psittacosis bodies)

Solution I

Phosphate buffer solution, 0.05 *M* (pH 7.0)	95 ml.
Formalin, neutral	5 ml.
Methylene blue, alkaline (Loeffler)	10 ml.

Solution II

Safranine O, 0.2-per-cent solution	25 ml.
Acetic acid, 0.1-per-cent solution	75 ml.

Procedure.—Apply solution I to the unfixed film for three minutes. Rinse in water and counterstain with II for thirty seconds. Wash in water, blot with filter paper, and allow to dry in air.

The psittacosis bodies are stained blue and the cells and background, pink.

Methylene blue, Unna's polychrome stain (28) (for leucocytes)

Methylene blue	1 gram
Potassium carbonate, $2K_2CO_3 \cdot 3H_2O$	1 gram
Water, freshly redistilled	100 ml.

Combine and heat for ten minutes at 60°C. Before use, dilute 1:3 with water.

Procedure.—See "Examination of Cerebrospinal Fluid," p. 519.

Newman-Lampert staining solution, No. 2 (29) (for direct microscopic count of milk)

Tetrachloroethane, technical (Eastman Kodak Co.)	40 ml.
Alcohol, 95-per-cent	54 ml.
Methylene blue	1 to 1.2 grams
Acetic acid, glacial	6 ml.

Combine the tetrachloroethane and alcohol and heat to a temperature not to exceed 70°C. Add the methylene blue and shake vigorously until the dye is completely dissolved. Cool and add the glacial acetic acid very slowly. Avoid heating the solution after the acid has been added. Filter and store in a tightly stoppered bottle.

Procedure.—See "The Examination of Milk and Cream," p. 603.

Pappenheim's stain—Barritt's modification (30, 31) (for Hemophilus ducreyi)

Methyl green, 0.1–0.5-per-cent solution chloroform treated	100 ml.
Alcohol, methyl	10 ml.

Pyronin G	0.5 gram, or more
Phenol	1 gram
Glycerol	20 ml.

Combine and let stand for twenty-four hours. Filter before use.

Methyl green, 0.1–0.5-per-cent solution chloroform treated

Methyl green	0.2–1.0 grams
Water	150 ml.
Chloroform	50 ml.

Shake in a separatory funnel for a few minutes. Discard the chloroform extract.

Procedure.—Fix the film with heat and allow the stain to act for one-half to one minute. Gram-negative bacteria are stained red. The nuclei of leucocytes are bright blue; the cytoplasm of polymorphonuclear cells is not stained but that of mononuclear cells is red.

Quensel's methylene blue-cadmium-sudan stain (32) (for protozoa)

Sudan III, saturated alcoholic solution (about 0.2 gram per 100 ml. 80-per-cent alcohol), decanted	20 ml.
Methylene blue, saturated solution (about 4 grams per 100 ml. water), filtered	50 ml.
Cadmium chloride, 10-per-cent solution	50 ml.

Mix the Sudan III and methylene blue solutions, filter, and add to the cadmium chloride. Let stand fifteen to twenty minutes, gently shaking occasionally. Filter and place the filter paper with the precipitate on another filter paper and leave overnight at room temperature to dry. Transfer the precipitate to a clean filter paper in a funnel and wash quickly by passing 25–30 ml. of water through it. Dissolve the precipitate in 250 ml. of water. Allow to stand a few days. If a fine crystalline precipitate is present, especially on the inner surface of the bottle, refilter.

Procedure.—See "Examination for Protozoa," p. 481.

Rees and Ecker's solution (33) (for platelet count)

Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$, 3.8-per-cent solution	100 ml.
Formalin, neutral	0.2 ml.
Brilliant cresyl blue	0.1 gram

Filter before use.

Procedure.—See "Examination of Blood," p. 505.

Sato and Sekiya's stain (34) (for peroxidase reaction)

Solution I

Copper sulfate crystals, $CuSO_4 \cdot 5H_2O$	0.5 gram
Water to make	100 ml.

Solution II

Benzidine	0.1 gram
Water to make	100 ml.
Hydrogen peroxide, 3-per-cent	2–4 drops

Test the hydrogen peroxide before use by adding to 2 ml. a few drops of a solution of potassium dichromate in sulfuric acid. If the peroxide is still active, a blue color will develop. Grind the benzidine in a mortar, gradually adding water to make 100 ml. Shake. Filter and add from 2 to 4 drops hydrogen peroxide. Store in a brown bottle. This solution will keep about a month.

Solution III

Safranine	1 gram
Water to make	100 ml.

Procedure.—Flood a freshly prepared blood film with solution I for one minute. Drain and cover with solution II for from three to eight minutes; wash in water. Counterstain with solution III for from one to two minutes. Wash and allow the film to dry.

Seller's stain (35) (for Negri bodies)

Basic fuchsin, saturated solution in absolute methyl alcohol (about 6 grams per 100 ml.)	2 ml.
Methylene blue, saturated solution in absolute methyl alcohol (about 1.5 grams in 100 ml.)	15 ml.
Methyl alcohol, absolute, neutral, acetone free	25 ml.

Make a trial stain by mixing the methylene blue and the methyl alcohol in a Coplin jar and adding 2 ml. of the saturated fuchsin solution. Macroscopically, the properly stained film, when held up to the light, should appear reddish violet in the thinner areas, shading into purplish blue in the thicker portions. If the thinner parts are bluish, add 0.5 ml. fuchsin. If this is not sufficient, keep adding 0.5-ml. amounts until the desired color is obtained. Store the mixture in a glass-stoppered bottle in a refrigerator to prevent evaporation and change in color. Allow to stand from three to four days to ripen before use. If evaporation takes place, the addition of absolute methyl alcohol will usually restore the proper balance.

Procedure.—See examination for evidence of rabies, p. 348.

Sodium hydroxide-glycerol solution (for fungi)

Sodium hydroxide, 10-per-cent solution	90 ml.
Glycerol	10 ml.

Procedure.—See "Examination for Incitants of Actinomycosis and Mycotic Disease," p. 466.

Swartz-Conant staining method (36) (for fungi)

Lactophenol-cotton blue (37, 38)

Phenol	20 grams
Lactic acid (about 85 per cent by weight)	20 grams
Glycerol	40 grams
Water	20 grams
Cotton blue (C_4B Poirrier)	0.05 gram

Dissolve the phenol in the lactic acid, glycerol, and water by gentle warming. Add the cotton blue.

Procedure.—Clear cutaneous scales, nail scrapings, or hair in sodium hydroxide-glycerol solution, then transfer to a small watch glass; wash two or three minutes with distilled water to stop the action of the sodium hydroxide; transfer to a glass slide, and cover with a drop of lactophenol-cotton blue; over this, place a round cover glass (22 mm.). Flatten the preparation, if necessary, by carefully pressing down on the cover glass. The epidermal cells are stained light blue; the granular protoplasmic content of the fungus is a deep blue.

To make semipermanent preparations, seal the edges of the cover glass to the slide with a cement such as Permount, a synthetic naphthalene polymer dissolved in toluene (Eimer and Amend). This procedure is facilitated, if a turntable is used. It is most important to remove completely any excess medium from around the cover glass before adding the sealing agent.

To prepare permanent mounts, stain the specimen in a few drops of lactophenol-cotton blue in a small watch glass instead of on the glass slide. Remove the stained specimen to a drop of clear lactophenol or glycerol placed in the center of a square cover glass (22 mm.); over this, apply a smaller round cover glass (18 mm.) and press down gently to flatten the specimen, if necessary. (Remove with blotting paper any mounting fluid that may have seeped from around the cover glass, since the success of the preparation depends upon having the edges entirely dry.) In the center of the round cover glass, place two drops of Clarite X (p. 547); over this, gently press a glass slide, which should then be lifted quickly and inverted. This procedure permits the mounting of the specimen between two cover glasses that are securely sealed by Clarite to the glass slide.

Tetrachrome stain (39) (for Donovan bodies)

Tetrachrome stain, dry (MacNeal)	0.5 gram
Alcohol, methyl	500 ml.

Dissolve the tetrachrome stain in the methyl alcohol. (The stain can be purchased in 0.5-gram amounts in capsules.) Shake thoroughly and leave at 37°C. for one or more days.

Procedure.—Cover the unfixed film preparation with stain and allow to act for five minutes. Then add an equal volume of water. Allow to stand for seven minutes. Wash with water. Drain and dry in the air.

Washburn's stain (40) (for peroxidase reaction)

Solution I

Benzidine	0.3 gram
Basic fuchsin	0.3 gram
Sodium nitroprusside, $Na_2Fe(CN)_5NO \cdot 2H_2O$, saturated solution (about 40 grams per 100 ml. water)	1 ml.
Alcohol, 95-per-cent	100 ml.

Dissolve the benzidine and basic fuchsin in the alcohol. Add the sodium nitroprusside solution. This preparation keeps several months.

Solution II

Hydrogen peroxide, 3-per-cent	0.3 ml.
Water	25 ml.

Prepare fresh daily.

Test the hydrogen peroxide before use by adding to 2 ml. a few drops of a solution of potassium dichromate in sulfuric acid. If the peroxide is still active, a blue color will develop.

Procedure.—Cover a freshly prepared thin film with 15 drops of solution I and allow to stand from one to two minutes. Add from 5 to 8 drops of solution II and allow it to remain from four to six minutes. Wash thoroughly in tap water; drain; and dry in air. Counterstain by Wright's method, p. 42, except that the treatment with the buffer should continue from thirty to forty-five minutes.

Wilhite's stain (modified) (41) (for Negri bodies)

Rosaniline	1.5	grams
Methylene blue	0.9	gram
Glycerol, neutral	125	ml.
Alcohol, methyl	125	ml.

Shake for several minutes. Allow to ripen for two weeks. Immediately before use, combine 10 drops of the stock solution with 20 ml. of potassium hydroxide, 1:50,000 aqueous solution.

Procedure.—See examination for evidence of rabies, p. 348.

Wilhite's stain—Schleifstein's modification (42) (for Negri bodies)

Rosaniline	1.8	grams
Methylene blue	1	gram
Glycerol, neutral	100	ml.
Alcohol, methyl	100	ml.

Shake for several minutes. Allow to ripen for two weeks. The mixture keeps indefinitely. Immediately before use, combine one drop of the stock solution with 1.5–2 ml. of potassium hydroxide, 1:40,000 aqueous solution.

Procedure.—See examination for evidence of rabies, p. 348.

Wright's stain (43–46)

Wright's stain, commercial preparation	0.1	gram
Alcohol, methyl	60	ml.

Mix the powder with the alcohol in an absolutely clean dry mortar. Allow to stand for twenty-four hours and then filter. Place in a brown glass-stoppered bottle and keep in the dark.

Procedure.—Without previous fixation, cover the film with Wright's stain. This requires approximately from 15 to 20 drops delivered with a capillary pipette. The stain can be prevented from overflowing by a wax-pencil mark at both edges of the film. Allow the stain to act for exactly one minute. With a second pipette add an equal number of drops of a 0.05 *M* phosphate buffer solution, pH 6.4. (Buffer solutions of other pH values may be necessary depending on the age of the film.) Allow to remain from five to fifteen minutes; the optimum time to be determined for each new lot of stain. Rinse with water keeping the slide in a horizontal position. Drain and dry in the air.

Ziehl-Neelsen stain (47, 48) (for acid-fast microorganisms)

Carbolfuchsin

Alcohol, 95-per-cent	100 ml.
Basic fuchsin	10 grams
Phenol, 5-per-cent solution	900 ml.

Dissolve the fuchsin in the alcohol. Add the phenol solution and allow to stand overnight. Pass through a diatomaceous filter candle to remove any acid-fast bacilli that may have been present.

Acid alcohol

Hydrochloric acid (sp. gr. 1.19)	3 ml.
Alcohol, 95-per-cent	97 ml.

Methylene blue, 0.1-per-cent solution

Methylene blue	0.1 gram
Water	100 ml.

Mix and filter.

Procedure.—Flood the films with carbolfuchsin and steam for five minutes. Blot dry or wash. Decolorize with acid alcohol. Wash in running tap water. Counterstain with methylene blue, 0.1-per-cent solution for one to two minutes. Wash in running tap water. Dry by blotting or draining.

Acid-fast microorganisms are red against a blue background.

Ziehl-Neelsen stain—modified (49) (for acid-fast microorganisms)

Carbolfuchsin

Alcohol, 95-per-cent	100 ml.
Basic fuchsin	10 grams
Phenol, 3-per-cent solution	900 ml.
Sodium chloride, 5-per-cent solution	10 ml.

Dissolve the fuchsin in the alcohol. Add the phenol solution and allow to stand overnight. Pass through a diatomaceous filter candle to remove any acid-fast microorganisms that may have been present. Add the sodium chloride solution and store at 37°C. since a precipitate forms at room temperature. The solution should keep for at least two months.

Acid alcohol

Nitric acid (sp. gr. 1.42)	2.5 ml.
Alcohol, 95-per-cent	97.5 ml.

Brilliant green

Brilliant green	1 gram
Sodium hydroxide, 0.01-per-cent solution	100 ml.

Procedure.—Cover films with the stain, steam for four to five minutes, and cool for two minutes or until a precipitate forms. Wash in tap water, decolorize in acid alcohol for from one to ten minutes. Wash in tap water followed by 95-per-cent alcohol for two minutes. Wash in tap water and counterstain with brilliant green solution or methylene blue, 0.1-per-cent solution, for one to two minutes. Wash and dry.

Acid-fast microorganisms are dark red rods against a green or blue background.

Zinc sulfate solution (50, 51) (for flotation of ova and cysts)

Zinc sulfate	330 grams
Water	1000 ml.

Determine the specific gravity. If greater than 1.180, correct by addition of water.

Procedure.—See "Examination for Protozoa," p. 482.

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CHAPTER 3

CARE AND BREEDING OF LABORATORY ANIMALS

Rabbits, guinea pigs, albino rats, and mice are bred and a supply maintained chiefly for general laboratory use. Small colonies of cotton rats (*Sigmodon hispidus hispidus*), ferrets (*Mustela putorius*), and a limited number of monkeys (*Macacus rhesus*) are kept on hand for special studies. Other species of animals and additions to the laboratory stock are maintained as required, or may be obtained from outside sources.

In order to reduce variations or irregularities in the results of tests, it is desirable that the previous history of the animals be known and that as far as practicable they be from a single source; it is essential that the animals be free from infection, vigorous, and healthy. A supply of such animals can be maintained only when their breeding and care are under competent supervision and when post-mortem examinations that include bacteriologic study are made routinely of animals dying in the breeding and stock quarters.

Breeding of laboratory animals is carried on at the laboratory farm where separate buildings with partitioned sections provide facilities for the separation of species and also of colonies of the same species. The number and size of the individual colonies depend upon the requirements and space available. To prevent heavy losses in case of epidemics, the number of animals in individual breeding colonies is limited to 3000 mice, 1000 guinea pigs, or 200 rabbits. Two or more breeding colonies of each species are maintained.

Special care is exercised in the selection of workers assigned to the animal quarters. In the care and handling of animals proper consideration and kindness on the part of all members of the staff are expected and required. An apparent infringement of this rule renders a worker liable to immediate discharge or suspension pending charges.

NEW STOCK FROM OUTSIDE SOURCES

Supplementary stock should be obtained directly from the breeder or from a source through which any infection that may be present in a shipment of animals can be traced. A contract for shipments at regular intervals may be advisable.

Upon receipt, place the animals in isolation quarters in clean, previously disinfected or sterilized cages or boxes. Attach a tag giving the number of animals, source, and date received. Except in emergencies, hold each shipment in quarantine for at least seven days before distribution. Should one or two animals of a lot appear ill when received or during the period of quarantine, isolate them at once. Make a post-mortem examination of those that die and as a rule continue the quarantine for at least one week after a death from a suspected or known infection.

ANIMAL QUARTERS AND CAGES

The animal quarters are divided into separate sections for (1) breeding animals; (2) animals recently received from outside sources; (3) stock for distribution; and (4) test and experimental animals. There are further subdivisions for animals returned for redistribution and for those likely to spread infection. Adequately equipped operating rooms are provided.

The animals quarters should be kept clean, orderly, properly ventilated, and free from vermin. A temperature of from 65° to 70°F. should be maintained for breeding mice and rats, and for monkeys, and from 60° to 65°F. for guinea pigs and rabbits. Higher temperatures may be advisable for test mice and rats when protective bedding is not used. Rabbits may be kept in lower temperatures; they should not be subjected to extremely high temperatures such as occur during the summer in exposed or poorly ventilated quarters.

Precautions against Flies.—The windows and outside doors of the animal quarters are screened. Electric screens and insecticides are used to destroy flies that have gained entrance. Cages are cleaned frequently; uneaten vegetables are not allowed to accumulate in them. When special precautions are required to prevent spread of infection by flies, cages or boxes are screened.

Precautions against Vermin.—Cages may become infested with cockroaches or bedbugs. Incoming shipments are therefore carefully watched and the boxes destroyed or sterilized. Cracks and crevices in which breeding may take place are eliminated as far as practicable. Prompt action is taken at the first evidence of infestation since delay allows dissemination and makes eradication more difficult. Effective results have been obtained by subjecting the entire room, while closed, to an insecticide delivered from an electric vaporizer, and then by immersing the infested cages in hot water (70°C. or higher). This process is repeated each week until there is no evidence of vermin.

Animal cages and boxes are made according to specifications. Metal cages of three sizes with five compartments arranged in vertical order, each provided with a noncorrosive metal or galvanized iron tray, are used in the stock and test animal quarters. A similar cage with four single or eight double compartments and expanded metal floor is used for rabbits and ferrets. Single-compartment cages with expanded metal or wire-mesh floors and nest boxes have been found most satisfactory for breeding rabbits. These are arranged in vertical order on metal racks with provisions for sliding trays. Breeding ferrets are provided with a combination wood cage and attached run with wire-mesh sides and top. An expanded metal floor is used for both cage and run. Each cage and run is divided by a longitudinal partition to form two single compartments. A smaller cage of similar design is used for cotton rats. Wooden or galvanized runs constructed to permit stacking are used for breeding and stock guinea pigs; wooden boxes that have wire-mesh covers with removable feed hoppers, for breeding mice. In the test animal quarters, glass jars are used for individual mice, boxes of noncorrosive metal with sliding wire-mesh covers for groups of mice and isolated guinea pigs, and larger boxes of galvanized iron for isolated rabbits.

Metal racks with open adjustable shelves are provided for animal boxes in the test quarters, wooden racks in the mouse breeding quarters. The monkey quarters have built-in cages of masonry and steel construction and an operating and service room.

Care of Cages

Use clean, thin, soft-wood shavings, cedar tow, or other material that has good absorbing qualities and is suitable for bedding. Make a layer about one and one-half inches deep. Fine, not chaffy, straw may be used in the deeper trays or compartments for guinea pigs and rabbits.

Clean all cages at least once a week, a cage compartment promptly after an animal has been removed. When necessary, cages are sterilized by immersing in a vat of hot water containing washing soda or placing in a steam chamber. Observe special care in cleaning cages occupied or vacated by animals suspected or known to have an infection that may spread to other animals. Sterilize cage with bedding or dampen bedding with one-per-cent cresol before removal. Vacated boxes and jars are sterilized routinely.

FEEDING OF ANIMALS

Since the condition and vitality of the laboratory animals depend largely upon the food they receive, a balanced ration is essential (1–3, a, b). Particular attention should be given to vitamin requirements. Constant supervision is necessary. The diets at present provided for the different species follows.

Guinea pigs and rabbits.—Water, salt, a concentrate mixture (Commercial rabbit pellets 3 parts, oats 3 parts, barley 1 part), and U. S. Standard Grade No. 1 or 2 second- or third-cutting alfalfa or clover hay are available at all times. Commercial rabbit feed in pellets containing a relatively large amount of ground alfalfa comprises at least one-half of the grain ration in the test animal quarters where hay is omitted to avoid dust. Green feed such as freshly cut alfalfa or clover in summer, and a mixture of chopped cabbage, carrots, and rutabagas in winter is given daily. Dried or decayed leaves are removed from cabbage, and roots are washed before chopping.

Mice.—Water and commercially prepared dog or laboratory animal feed in pellet form are available to breeding mice at all times. Small feedings of cabbage, lettuce, or other green leafy vegetables are given to breeding and stock mice two or three times a week. Dried bread and a grain mixture (oats 1 part, whole wheat 1 part, and buckwheat 1 part) may be wholly or partially substituted for the commercial pellets when fed to stock mice.

Albino rats.—The diet fed to breeding mice is given with the addition of uncooked beef or liver twice a week. Milk is fed two or three times a week.

Cotton rats.—Water, commercial animal feed in pellet form, and a grain mixture (equal parts oats, whole wheat, and buckwheat) are available at all times. Cabbage, carrots, apples, and sunflower seed are fed three or four times a week.

Ferrets.—Crushed whole wheat cooked with cabbage, carrots, or alfalfa leaves is fed daily with diluted milk (equal parts whole milk and water). Uncooked horse meat is fed four times a week and uncooked liver twice a week. A limited amount of commercially prepared animal feed in pellet form is fed daily.

Monkeys.—Monkeys are supplied with water and fed twice a day. The morning feeding is bread and milk; the afternoon, which is varied each day, consists of fruit (apples, oranges, bananas, prunes) and vegetables (cooked sweet or white potatoes, carrots, turnips, cabbage). Unroasted peanuts are provided three or four times a month.

Special Equipment

Water bottles.—Bottles with rubber stoppers and glass tubes about 10 mm. in diameter and constricted at the delivery end to about 2 mm. Quart or pint milk bottles are used in quinea-pig and rabbit cages; 6- or 8-ounce nursing bottles in mouse cages. They are supported by a wire holder attached to the cage. Bottles of suitable size inverted and held in place by the delivery tube, which passes through the mesh cover, are provided for test animal boxes and jars. Bottles of approximately 16-ounce capacity are used for the mouse breeding boxes which have a metal bottle-support on the outside of the cover.

Grain crocks.—Heavy earthenware crocks, 5 inches in diameter and 4 inches high, with glazed surface. They are washed and sterilized in the bottle-washing machine.

Hay racks.—Galvanized iron with hinged cover and open wire front (1- by 4-inch mesh).

Bottle-washing machine.—A standard two-compartment milk-bottle washing and sterilizing machine of pressure jet type. Standard milk-bottle crates for quart and pint bottles and special crates for smaller bottles are used. All bottles and delivery tubes and stoppers are washed and sterilized before each refilling.

Scales.—Three types of scales are used to weigh animals: (1) Springless scale with extended tare beam and graduated on chart to 524 grams by 2 grams and on tare beam to 1 kilogram by 5 grams, for mice, guinea pigs, and light-weight rabbits. Mice are weighed in a receptacle at the end of the extended tare beam and the readings obtained on the scale are divided by 10. (2) Balance scale with tare beam, capacity 13.5 kilograms by 5 grams for rabbits. (3) Spring scale with dial, capacity 250 grams by 1 gram, and removable pan, for mice and rats.

PREVENTION AGAINST SPREAD OF INFECTION

Production of laboratory animals free from bacterial diseases is dependent upon maintaining breeding colonies free from the incitants of these diseases. Through the process of selection and elimination in a series of successive breeding colonies with known family histories and records of findings at autopsy, it has been possible to develop colonies free from some or all of the infections common among these animals.

Prevention, in so far as possible, of the entrance of the inciting agent is the first precaution; next, the early recognition and eradication of any infection that has gained entrance. To accomplish this requires careful autopsies and bacteriologic examinations of the animals that die.

Animal breeding colonies are maintained at the farm where physical separation from animals used in tests or animals received from outside sources can be provided. This separation is further maintained between species of animals and between different colonies of the same species. Infections of low pathogenicity for one species may be transmitted to another species with disastrous results. Maintaining two or more breeding colonies for each type of animal insures against the loss of production in the event that infection gains entrance in one of the colonies.

To prevent carrying infections from one group to another, caretakers, in so far as possible, are assigned to individual groups, or a change of clothing is provided. As the workers enter the quarters for breeding animals, they step on a mat soaked with disinfectant.

Caretakers are trained to observe animals closely and to report symptoms of illness or disease. Sick animals are promptly isolated or destroyed as directed. If the bacteriologic examination reveals that an animal suffered from an infection likely to spread to other animals, immediate steps are taken to isolate, or destroy if advisable, all animals in the compartment or the tier from which the animal was removed. In the breeding quarters, isolation may be extended to animals in the parent and offspring cages or to a whole section of the colony.

DISPOSAL OF DEAD ANIMALS. POST-MORTEM EXAMINATIONS

Careful necropsies are made of all dead breeding animals and of dead animals from new stock held in quarantine. If only occasional deaths in scattered cages occur in the stock quarters, the animals are not always autopsied. At the first indication of the possible spread of infection in one or more cages, post-mortem examinations are made immediately. A permanent record is kept of necroscopic and bacteriologic findings.

DISEASES OF SMALL ANIMALS

A knowledge of the normal animal (4–11) as well as of the diseases of laboratory animals (12–23) is essential not only for the proper management of breeding and production but also in the interpretation of the results of tests in which animals are used. Brief descriptions of certain bacterial and parasitic infections that have been recognized among the small animals at this laboratory are included as an aid to workers responsible for their care or use in routine tests. More complete information may be obtained from reference books or the extensive reports found in the literature. Anatomic specimens showing the pathologic lesions of many of the diseases of laboratory animals and slide preparations of normal tissues are available in the museum for reference and study.

Acute and chronic respiratory and septicemic infections may occur at

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any time among laboratory animals although they are usually less prevalent during the summer months. Some animals may be carriers of the incitants of these infections throughout the year. Clinical evidence in many of the specific diseases of small laboratory animals may not be manifest or may be of uncertain diagnostic significance. Certain incitants are characteristically associated with specific lesions.

Streptococcus.—Hemolytic streptococci, group C, have frequently been the incitants of enzootics or epizootics of pneumonia and lymphadenitis in guinea pigs. Pneumonia, more prevalent during the winter and spring months, may cause a high mortality among the breeding or stock animals while test animals weakened by the presence of infection at the time of inoculation may succumb, thereby causing irregularities in the results of tests. The principal lesions are those of pneumonia, with or without pleurisy and pericarditis. Cultures from lung lesions are taken on blood-agar plates (*F43A*). During an outbreak, the streptococci may be recovered from the nares of a large percentage of apparently healthy contact animals.

Lymphadenitis in guinea pigs is a chronic disease involving the cervical or other lymph glands and is manifested by encapsulated abscesses that are usually not noticed until they have attained a considerable size. The infection may be produced experimentally by inoculation of the scarified skin with streptococci of group C recovered from guinea pigs. The glandular involvement may occur in an individual animal without the presence of pneumonia, but it is more frequently found among groups of animals showing the respiratory disease. Streptococcus lymphadenitis has not been seen at this laboratory in colonies of guinea pigs free from streptococcus respiratory infections.

While the guinea pig is particularly susceptible, other laboratory animals may die from an infection with streptococci of serologic group C. In one instance, an outbreak of respiratory infection was observed among a small group of mice. In 1940, a limited outbreak of hemolytic streptococcus infection among a group of stock rabbits was concurrent with a similar outbreak among guinea pigs in the same quarters.

Staphylococcus.—An infection incited by *Staphylococcus aureus* was associated with an enzootic in the rabbit colonies during 1939 and 1940. The disease was characterized by single or multiple subcutaneous abscesses confined to a limited area or disseminated. Abscesses in the mammary glands were common among breeding rabbits. Respiratory infections and abscesses of the lungs were occasionally seen. To prevent spread of the infection, the prompt detection and removal of affected animals and the isolation of contacts are essential. Complete elimination of the infection was obtained after replacement of the breeding colonies

Pneumococcus.—Sporadic cases of pneumonia or septicemia due to the pneumococcus have occurred among guinea pigs, occasionally in rabbits. Strains recently recovered from necropsies have been classified as type 19. A fibrinous exudate may be found in the thoracic or peritoneal cavities or in both.

Bacterium bronchisepticum.—This microorganism is a common cause of respiratory disease among guinea pigs and rabbits. Its high degree of infectivity and low virulence have frequently lead to a relatively wide dissemination of the microorganism among a group of animals before its presence was detected. The disease is definitely seasonal, being most prevalent during the winter and spring months. It is probable that individual animals carry the incitant in the upper respiratory tract throughout the year. Nasal cultures taken on blood-agar plates from apparently healthy contacts have shown a high percentage of infected animals. Guinea pigs may for several days show moist nose, arched back, roughened coat, and rapid breathing. At necropsy, marked congestion or consolidation of the median lobe or lower borders of other lobes is usually found.

Bact. bronchisepticum has been recovered from the nares of live rabbits and at necropsy from the bronchi or lungs of animals with or without evidence of snuffles or other symptoms of respiratory disease.

Pasteurella septica.—Microorganisms of the hemorrhagic septicemia group have been found as the incitants of acute or chronic respiratory infection, subcutaneous abscesses, and middle ear infections in rabbits. The infection may progress rapidly to an acute fatal pneumonia or follow a more chronic course. Pleurisy and pericarditis may be associated with the pneumonia. In the chronic form, infection is frequently confined to the upper respiratory tract with symptoms of varying degrees of snuffles. This type as well as the more chronic middle-ear infections and subcutaneous abscess formations probably represents a variation in the susceptibility of the host.

Infections are most prevalent from October to April and are very difficult to control in crowded quarters. Cultures from the nasal passages of live animals or from bronchi or lungs at death are taken on blood agar. Immediate isolation of contacts, sterilization of cages, and nasal cultures for detection of carriers may assist in controlling the infection.

In 1931, an outbreak with high mortality occurred among a group of approximately 60 guinea pigs. The post-mortem examination in most instances showed peritonitis or pleurisy and a fibrino-purulent exudate covering organs of the abdominal or thoracic cavity or both. Pericarditis, pneumonia, and, in mature females, marked congestion of the uterus with evidence of abortion were frequently seen. Cultures on blood-agar plates showed small greyish-white colonies which tended to be confluent and in patches. Cultures on Endo agar (*F52A*) gave an inhibited growth. The incitant, on the basis of later tests, appeared to be *Pasteurella septica*. It was pathogenic for guinea pigs and mice when injected intraperitoneally. Reports of a similar disease have been made by Roth (18), Wright (19), and others.

Pasteurella pseudotuberculosis.—Infections incited by *Past. pseudotuberculosis* have been reported as occurring in animals and birds, also in persons. The lesions in guinea pigs may resemble those of tuberculosis, plague, tularemia, glanders, and infection incited by *Brucella abortus*. Besides the so-called classical type of infection characterized by diarrhea and emaciation, an acute septicemia and a glandular type have been described. One outbreak has occurred among a group of guinea pigs at the farm. The characteristic findings at necropsy were peritonitis or pleurisy and focal necroses with abscess formation in all viscera. Heavy growth was obtained in cultures from the spleen and lungs.

Mycobacterium tuberculosis.—Monkeys and other animals may become infected and develop pulmonary or generalized tuberculosis. Monkeys are extremely susceptible; new shipments should be kept under strict quarantine and animals from different shipments preferably in separate cages.

Salmonella.—Microorganisms of the salmonella group frequently incite severe epidemics among guinea pigs and mice (mouse typhoid). The term "pseudotuberculosis" associated with these infections in guinea pigs should be restricted to the specific disease incited by *Past. pseudotuberculosis*. The symptoms in guinea pigs are roughened coat, loss of appetite, and inactivity. The course of the disease may be so rapid that death occurs before symptoms are observed. In mice the roughened coat is pronounced and diarrhea is usually present. Necropsy findings vary. Enlargement of the spleen, the most constant finding, may be slight or very marked. In cases of long duration, multiple abscesses are frequently found in the spleen, less often in the liver. There is evidence that some animals may recover and become carriers. The strains isolated in this laboratory have with few exceptions fallen into two distinct groups. While *Salmonella enteritidis* has frequently been isolated from mice and guinea pigs, more severe epizootics have resulted from infection with *Salmonella typhimurium*. The sudden appearance of these infections in scattered cages within colonies which for years have apparently been free from them has emphasized the ever-present danger of introducing the infection through hay or feed that has been exposed to wild rodents.

Ringworm.—Ringworm may occur in laboratory animals. Workers caring for infected animals should wear gloves. Treatment consists of applications to the affected areas of tincture of iodine or an effective ointment.

A dermatitis, apparently caused by a fungus, is found among laboratory mice. It may be confined to the tail or appear on the head and elsewhere. Infected animals are either destroyed or treated by dipping on alternate days in a 1:500 solution of potassium permanganate.

Coccidiosis.—Two types of coccidiosis are common among rabbits, frequently with high mortality among the young, growing animals. In the intestinal type, diarrhea and emaciation are the most pronounced symptoms. The mucosa is inflamed and *Eimeria perforans* may be found in great numbers in the lumen of the intestine or the feces. Should the animal recover from the acute infection, the disease may become chronic and continue for a variable period, probably dependent upon the amount of damage and response of the tissues.

The hepatic type is characterized by focal necrosis or abscess formation with inflammation and frequently a greatly enlarged liver. *Eimeria stiedae* may be recovered from active liver lesions or the gall bladder. In animals that have recovered from an acute infection the liver lesions may have become fibrotic, and microscopic examination of crushed lesions show few or no oocysts.

Use of elevated expanded-metal or wire-mesh floors, sanitary measures, and removal of infected breeders and their offspring have aided in eliminating or controlling infection.

The presence of coccidia in large numbers in the intestines of guinea pigs has been found associated with an enteritis and diarrhea.

Intestinal parasites.—Larval forms of tapeworms are found encysted in the livers of mice and rats. The cystic stage of a dog tapeworm, *Taenia pisiformis*, may be found in the liver or mesentery of rabbits. Mature cestodes and nematodes have been observed in the gastrointestinal tract of mice.

Mites.—Ear mange caused by a mite, *Psorptes cuniculi*, is not uncommon among rabbits. The infestation begins on the inner surfaces of the external ear where thick brown deposits may be found often filling the external canal. Infested animals are isolated and the ear treated with a solution of bichloride of mercury 1 part, glycerol 100 parts, and 50-per-cent alcohol 200 parts; or 1-per-cent cresol in a mixture of liquid petrolatum 3 parts and kerosene 1 part.

Lice.—Lice may infest any of the laboratory animals. Animals in shipments from outside sources should be examined and if infested treated immediately. Treatment consists of repeated application of a dusting powder containing derris root, worked well into the hair. DDT preparations may be found to be more effective.

BREEDING OF LABORATORY ANIMALS

Rabbits, guinea pigs, and mice are bred routinely for laboratory use (2, 24–28). The present breeding colonies have been developed from a limited number of carefully selected stock. When starting a colony, a small number of animals are selected, divided into groups, and inbred within the groups until the absence of infection is relatively assured. Subsequent breeding stock is selected with care. Records of family histories and production are maintained. Individuals or families whose productivity is low or that demonstrate other undesirable characteristics are eliminated.

Rabbits.—Period of gestation from thirty to thirty-three days. The percentage of conceptions per service usually somewhat lower during late summer and early fall months. Usual litter from four to eight young. Three to five litters usually obtained in a year. Mate when from six to ten months old. Keep each breeding rabbit in a separate compartment or cage and assign from four to eight females to a single male. When mating, always take the female to the male's cage. Check mate at from ten to fourteen days following service if desired. Provide nest box and bedding for a nest one week before parturition. Do not disturb or handle young in the nest. Tattoo and remove the young when from four to six weeks old. Place males and females in separate cages. Discontinue breeding when rabbits are from three to four years old.

Guinea pigs.—Period of gestation from sixty-eight to sixty-nine days. Usual litter from two to four young. Four or five litters obtained in a year. Mate when from four to six months old. Keep one male with from six to nine females. When a new run is to be started, select young females from a single parent compartment or run and the male from another. Remove the young for stock when from three to four weeks old and place males and females in separate runs. Discontinue breeding when guinea pigs are from two to three years old.

As a precaution against the spread of infection, large guinea-pig breeding colonies are divided into four or more groups with no cross breeding between them.

Mice.—Period of gestation from nineteen to twenty-one days. Usual litter from four to seven young. Four or five litters may be expected from a breeding female. Mate when from two and one-half to three months old or place the male with selected females when weaned. Place one male in a box or compartment with from eight to ten females. Select the females and the male from separate parent groups as described for guinea pigs. Remove the young when four weeks old and separate according to sex. Discontinue breeding when a mouse is from ten to twelve months old.

Rats.—Period of gestation from twenty-one to twenty-eight days. Average litter from six to eight young. From five to seven litters may be expected from a female. Mate when from three and one-half to four months old. Place one male with from one to three females or two with from four to seven. Remove the young when from four to five weeks old and separate according to sex. Reproduction starts to decline when a rat is about twelve months old and usually ceases when it is between fifteen and sixteen months old.

Ferrets.—Estrus occurs in spring and again in summer. If not bred, estrus period persists for a long time. Ovulation is stimulated by coitus and occurs approximately thirty-hours later. Period of gestation from forty-one to forty-three days. Mate females when approximately one year old. Observe mating carefully, since certain males refuse to breed. If desired, use the same male or different males on successive days. Keep each ferret in a single cage or compartment and provide nest box and bedding. Do not handle or disturb the young in the nest. Remove the young when from two to three months old.

DISTRIBUTION OF ANIMALS FOR LABORATORY TESTS

Routine laboratory tests and procedures that require animals of specified weights or ages are:

	Guinea Pigs *grams*
Leptospira icterohaemorrhagiae, isolation and identification	100 to 150
Standardization of diphtheria and botulinus toxins and antitoxins; detoxification of diphtheria toxin (subcutaneous method)	230 to 280
Standardization of diphtheria toxins and toxin dilutions for intracutaneous test of susceptibility (Schick)—(Subcutaneous method)	250 to 280
Corynebacterium diphtheriae virulence tests (intracutaneous method) (white)	250 to 350
Isolation and identification of microorganisms	250 to 350
Determination of antigenic value of fluid diphtheria toxoids (subcutaneous method)	270 to 320
Standardization of tetanus toxin, antitoxin, and toxoid (subcutaneous method)	330 to 380
Tests of harmlessness of products for human use	350 to 500
Standardization of diphtheria toxin and antitoxin (intracutaneous method) (white)	350 to 450
Determination of antigenic value of precipitated diphtheria toxoids (subcutaneous method)	470 to 520
For complement in serodiagnostic tests (previously used or normal)	Over 600 preferably

Demonstration of spirochetes and pathogenic yeasts and molds	**Rats** *adult*

	White mice *grams*
Pertussis vaccine (potency tests)	8 to 11
Typhoid vaccine (potency tests)	14 to 16
Demonstration of Negri bodies (rabies)	10 to 15
Pneumococcus and streptococcus virulence tests	16 to 22
Standardization of antimeningococcus serum	16 to 20
Standardization of antipneumococcus serum	18 to 22
Tests of harmlessness of products for human use; isolation and identification, and determination of toxigenicity of microorganisms	20 to 30

	Rabbits *grams*
Production of various immune sera	Over 1800
Production of antipneumococcus sera	Over 2500
Production of hemolytic amboceptor	Over 3000
Standardization of streptococcus toxin and antistreptococcus serum; streptococcus toxigenicity tests and toxin-antitoxin neutralization tests (intracutaneous method) (1 year or older, white skin)	Over 3000

Select animals preferably from the same source for a test; otherwise, keep those from each source separate and identified.

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CHAPTER 4

CARE AND USE OF LARGE ANIMALS

Horses are used for the production of antitoxic and other immune sera. Their blood and sera are also used in the preparation of culture media and for other purposes. Mules have proved satisfactory for the production of certain antitoxic and diagnostic sera. Sheep furnish red blood cells for use in complement-fixation tests and blood for media. Goats are employed mainly in tests of potency of streptococcus toxins. The care and treatment (1–3) of all large animals are under veterinary supervision. The majority of the larger animals are maintained at the Laboratory Farm as are breeding units for small animals.

Each animal of the different species is numbered consecutively in the order of receipt. Horses have the number branded on the right forward hoof. Goats and sheep are identified by a number tattooed on the inner surface of the ear, or by a metal tag attached to the collar. Sheep may be further identified by painting the number on a clipped area on the side with oil of tar or a nontoxic paint.

HORSES

The evidence available indicates that the breed of horse used is of no special importance in the production of antitoxic and other immune sera. It is essential, however, that the horse be healthy and in good condition. Vigorous, mature animals not over twelve years of age, weighing 1100 pounds or more, are preferred. An old horse may respond well to immunization but its period of usefulness is usually shorter.

Horses are purchased from reliable dealers and private owners. A limited number are transferred from other State departments and a few are gifts. Horses with certain defects which lower their market value may, if healthy, be entirely suitable for purposes of immunization.

Examination and Quarantine

Horses are quarantined on receipt and kept under daily observation for at least sixty days. Each animal is tested with mallein for evidence of glanders; temperatures are taken daily, and at least one complete blood cell count is made during the period of quarantine.

Immunization against Tetanus

The horses are actively immunized against tetanus soon after receipt. Two initial doses, 10 and 20 ml. respectively, of tetanus toxoid to which has been added sufficient 20-per-cent aluminum-potassium-sulfate solution to give a 0.5-per-cent concentration are injected subcutaneously thirty days apart. A single dose of 20 ml. is then administered once each year. Additional toxoid is given, if indicated, following injury.

Quarters and General Care

Stables.—Stable units with straight stalls, an ample number of box stalls, operating and service rooms, and facilities for individual and group isolation are provided at the Central Laboratory and at the Laboratory Farm. Rotation of pastures is practiced to assist in parasitic control and to increase the quality and quantity of the grazing.

The horses are stabled at night and turned into pasture or paddocks during the day if the weather and schedules of immunization and bleeding permit.

Feeding of Horses (1).—It is difficult to prescribe definite rules for feeding horses, since individual requirements may vary. In general, give daily in two feedings from 1 to 1½ pounds of U. S. Grade No. 1 mixed hay containing timothy and from 15 to 30 per cent of clover or alfalfa per 100 pounds of weight. Give approximately ⅓ of the daily roughage at the morning feeding, the balance at the evening feeding. If alfalfa or clover hay is substituted, feed somewhat less. Considerably less roughage is required when adequate pasturage is available. Gauge the amount of the concentrates to be given largely upon the general condition of the animal and the quality of the roughage (ordinarily somewhat less than 1 pound per 100 pounds of weight). Horses doing hard work require proportionately more. Oats have been adopted as the standard concentrate for horses, although other grains are suitable when properly fed. Corn is frequently substituted and supplemented with linseed or soybean-oil meal. Wheat bran is given at two or more feedings weekly. Crushed oats may be fed horses unable to masticate whole grains. Salt in brick form and water supplied by a drinking fountain with nose-control valve are available in each stall.

Temperatures, Observations, and Special Examinations.—The temperatures of all horses are taken each morning and their general appearance and condition observed. Temperatures are also taken after intravenous injection of live microorganisms, usually after from three to four hours, and in case of reactions following injection of killed vaccine. In case of marked and rapid elevations, over 104°F., additional temperatures are taken at intervals of from one-half to one hour until the maximum temperature has been obtained. Special clinical and laboratory examinations are made and treatment given as indicated. All the horses are tested once a year for evidence of glanders. Fecal examinations are made to determine the degree of parasitic infection; treatment for gastrointestinal parasites is given when indicated.

Rest Periods

Rest periods of from one to three months a year during the immunization of a horse are sometimes desirable. The potency of the serum and the general condition of the animal are taken into consideration.

A horse producing potent serum can be rested and on reimmunization produce serum of approximately the same or higher potency. In some instances it may be advisable to obtain all serum possible by bleeding out or to take two or three consecutive bleedings and hold the horse for reassignment. As a rule, while a horse is producing potent serum, injections and bleedings are continued, barring special disabilities, until two or more tests have shown a considerable decline in potency.

Reassignment or Disposal of Unproductive Animals

Horses that fail to produce sera of satisfactory potency after a sufficient period of immunization with one type of antigen may be bled for serum to be used in the preparation of certain culture media or after a short rest period reassigned for the production of immune serum of another type. Animals not suitable for further immunization are destroyed, usually by exsanguination while under general anesthesia. Horse meat, if suitable, is supplied to the media department.

Post-Mortem Examinations

Post-mortem examinations are performed on all horses (4). An examination is always made of the abdominal and thoracic viscera. The brain and cord are removed and examined when indicated. For the treatment of tissue for microscopic sections and of gross specimens, see chapter on histologic procedures, p. 539. Slide preparations are made and cultures taken when there is evidence of an infectious process.

INJECTION AND BLEEDING

The operating rooms are provided with stocks closed on the sides and front. Aseptic precautions are observed in all procedures. To prevent errors, the information on the direction slip and on the bottle that contains the material to be injected, and the number on the horse's hoof are checked aloud by two persons. A similar procedure is followed at the time of a bleeding.

Apparatus for Subcutaneous Injection by Air Pressure.—A bottle to contain about 500 ml. with 25-ml. graduations is used when over 40 ml. are to be injected by positive pressure. It is closed with a rubber stopper through which pass two right-angle glass tubes and one short straight tube. To the latter is attached rubber tubing (2 inches long) with a clamp which is released to permit the escape of air when the bottle is being filled. The second tube, which reaches just below the stopper, is connected with the stock bottle of toxin or toxoid when the injecting bottle is being filled and with the pressure pump when the material is being injected. The third tube which reaches the bottom of the bottle is fitted with pressure tubing (15 inches long) to which a needle (17 gauge, 2½ inches long) is attached. The bottles and attachments are sterilized by boiling in water or by autoclaving just before use. A screw clamp with two hinged arms is used to hold the stopper in place. On bottles with a flange insufficient to hold the clamp, a metal collar is substituted.

Apparatus for Intravenous Injection by Gravity.—A special graduated cylinder (capacity 25, 50, or 250 ml.) or a pyrex glass filter tube is used as a funnel. The cylinder is connected with a needle (15 gauge, 1½ inches long) by two 6-inch lengths of rubber tubing joined by glass tubing which serves as a window. It is sterilized by boiling in water or by autoclaving just before use.

Bleeding Jars (for Serum).—The outfit consists of a cylindrical battery jar of pyrex glass, a metal cover with turned-down edge, and a metal weight. The weight is provided on the under side with short pegs or teeth for engaging the clot, on the upper surface with a stud which passes through a central hole in the cover and is supported by a cotterpin. The weight is released by a string attached to the cotterpin and extending down over the side of the jar. A second hole in the cover and one in the weight provide for a rubber stopper and inlet tube. A close-fitting band of "silence" cloth or Canton flannel is fitted about the top of the jar under the turned-down edge of the cover. After assembling, the top of the jar is covered with paper which is tied firmly below the edge of the metal cover.

Bleeding Tubes (for Serum).—Tubes of pyrex glass, 2⅝ inches outside diameter and 40 inches long, wall thickness 1.5 to 3.5 mm., graduated at 2 and 2½ liters. Bottom rounded, open end reinforced and fire polished. Weights (approximately 3 pounds) 2¼ inches in diameter and 3 inches high, made of iron coated with tin and provided with a staple. They are sterilized with a string attached which is used when the weight is dropped into the tube after the clot has formed.

Bottles for Defibrinated Blood.—Wide-mouthed bottle (500 ml.) containing from 30 to 40 grams of 4-mm. glass beads and fitted with a rubber stopper through which pass an inlet tube connected by means of rubber tubing with a needle (13 gauge, 2 inches), and a cotton-plugged air-exhaust tube. The needle is placed in a tube, fitted with a cotton plug, which is tied to the bottle.

For larger amounts, a 2-liter flask is prepared in a similar manner, from 70 to 80 grams of glass beads and a cannula (5 mm. diameter, 3 inches long) being used.

Preparation of Animal.—As a rule a horse is not placed in pasture or exercised before an injection or bleeding. Feed is withheld for a period of at least four hours before bleeding. Horses with a temperature over 101°F. are not bled for therapeutic serum. A vacuum groomer is used routinely; when necessary, horses are brushed down immediately before entering the operating room.

Stocks: Posts with attached 7-inch cleats for ropes and horizontal bars of 3½ inch OD pipe; sides and front door of ¼-inch iron plate. The stocks occupy a floor space of 6 by 3 feet. The head post is placed 18 inches in front of the stocks in line with one side so as to give unobstructed access to the side of the horse's neck, usually the left.

Subcutaneous Injections

Injections of bacterial toxins or toxoids are given subcutaneously.

Small Volumes.—For doses of less than 40 ml., pipette under aseptic precautions the required volume into a sterile container. Add aluminum potassium sulfate or calcium chloride solution when required. Mix thoroughly. Then draw the toxin or toxoid into a graduated syringe. When the dose is less than 20 ml., make up to that volume with salt solution.

Volumes Greater Than 40 ml.—For volumes greater than 40 ml., use a sterile 500-ml. graduated bottle and inject by positive pressure. With aseptic precautions, insert a siphon with stopper and connections into the stock bottle, cover the stopper with cotton dampened in 2-per-cent cresol, and tie securely in place. Keep the same siphon in a bottle until the material is exhausted. Protect the outer tip to prevent contamination. If a siphon is to be transferred to another bottle, rinse it with water until clean and boil for fifteen minutes. Bring the stock bottles from the cold room as needed and avoid unnecessarily exposing the preparation to light or heat. Before using, examine the material carefully for physical evidence of contamination.

Assemble the injecting bottle and siphon connections. After sterilization insert the stopper firmly and clamp in place. To transfer the toxin or toxoid, connect the rubber tubing on the inlet tube of the stock bottle with the outlet tube of a Woulff bottle containing 2-per-cent cresol, and the outlet tube with the inlet tube of the injecting bottle. Connect the Woulff bottle with an air-pressure outlet and allow sufficient air to pass through to force the required amount of material into the injecting bottle. Add aluminum potassium sulfate or calcium chloride solution through the inlet tube when required. Mix thoroughly.

Preparation of Sites.—Prepare the sites of injection with an electric clipper with close-cutting "surgical" blades or by shaving areas on each side of the horse. Avoid injecting over the scapula, too near the vertebral column, or lower and further back than an extended line formed by the curvature of the last rib. Gauge the number of sites by the amount of toxin or toxoid to be given. Disinfect each with 2-per-cent cresol compound followed by 70-per-cent alcohol and tincture of iodine diluted with an equal part of alcohol.

Injection of Toxin or Toxoid.—If the amount to be injected is less than 40 ml., use a syringe. Inject amounts greater than 40 ml. by air pressure. The air passes through a cotton filter and is maintained at 10 pounds pressure. Connect the inlet tube of the injecting bottle with the pressure line from the air filter. Hold the needle over a waste receptacle, apply pressure until a steady flow of toxin is obtained. Stop the flow by sharply doubling the tube. Keeping the bottle near enough to the horse to prevent the possibility of breaking the connections should the animal struggle, insert the needle subcutaneously into one of the sites prepared for injection and release the bend in the tube until the desired amount has entered, usually not more than 25 ml. If the material is precipitated, shake the injection bottle frequently to maintain a uniform suspension. Disinfect the needle with 2-per-cent cresol compound and repeat the procedure until the desired dose has been given. After each injection paint the site with iodine.

Injections of Agar and Live Microorganisms (5).—Prepare an area from 2 to 4 inches in diameter on the horse in or near the saddle region. Melt 1.75-per-cent beef-infusion agar (*F33*) by heating in a water bath. Cool and draw into a syringe 40 ml. when the agar is not too fluid but before it gels (about 45°C., which may be determined by a control bottle of agar equipped with a thermometer). Then insert the needle from 1 to 1½ inches under the skin and inject. After about five minutes, inject the culture into the agar and move the point about to distribute the culture.

After an abscess has broken and is discharging, cleanse frequently the area around and below the site with 2-per-cent cresol compound. When toxin is to be injected, select areas on the opposite side of the horse or at some distance from the abscess. Rubber gloves and goggles should be worn by workers while injecting cultures or treating abscesses.

Intravenous Injections

This method is used in the routine production of immune sera, such as antimeningococcus and antipneumococcus, in which whole cultures or suspensions of bacteria in salt solution are injected. Injections are usually made by the gravity method; a syringe is sometimes used for small volumes.

Killed Microorganisms.—Always shake the bottle immediately before giving the dose. Depending upon the kind of microorganism and the schedule, the suspension is injected as received or diluted with salt solution to 200 ml. immediately before the injection.

Live Microorganisms.—Maintain the cultures at approximately incubator temperature until just before injecting. Give the dose in one injection or in two an hour apart depending upon the schedule. Cultures of meningococci should be injected without delay after being suspended in salt solution.

Procedure.—Prepare a site, as for subcutaneous injection, over the jugular vein. Moisten the hair on the entire side of the neck with 2-per-cent cresol compound. Occlude the jugular vein by pressing the thumb of the left hand firmly into the jugular groove below the site of injection. Holding the needle and tubing with the right hand and supporting the cylinder on the right forearm, force the needle pointed downward through the skin into the vein with a sudden thrust. When it is in the vein, blood will pass through the tubing into the cylinder and recede quickly when the pressure on the vein is removed. While the tubing is filled with blood, have an assistant pour into the cylinder from 20 to 30 ml. of salt solution followed by the inoculum. Control the rate of flow by raising or lowering the cylinder or by pressure on the rubber tubing. When the cylinder is nearly emptied, pour in 20 or 30 ml. of salt solution. Take care that air is not drawn into the vein. When the last of the salt solution has reached the glass "window," pinch the rubber tubing below it to stop the flow and quickly remove the needle. Paint the site with iodine. Sterilize contaminated glassware by boiling or autoclaving. During the injection, observe the animal closely for symptoms of reaction and decrease the rate of injection or stop the injection if advisable. In case of very severe or other alarming symptoms, inject epinephrine intravenously without delay or camphorated oil intramuscularly, or both, as indicated, the dosage depending upon the size of the animal and the severity of reaction.

Severe reactions following injections may be partially or entirely controlled by giving one-fourth of the dose followed by the remainder one-half hour later if no reaction occurs, also by the addition of from 0.75 to 1.5 ml. of epinephrine solution (1:1000) to the culture at the time of injection.

Bleedings

In order to obtain a clear serum, the morning feeding is withheld until after the bleeding has been taken.

Horses are bled from the jugular vein. Rigidly aseptic precautions are observed to prevent contamination. From seven to nine liters of blood are taken for a large bleeding, the amount depending to some extent upon the size and condition of the horse. Plasma is obtained by drawing the blood directly into sodium citrate solution; serum, by allowing the blood to stand until the clot is formed. The yield of serum is increased by exerting pressure on the clot. Smaller amounts are taken for defibrinated blood. Samples (trial bleedings) for tests are taken during the course of immunization.

Bleeding for Serum.—Boil the cannula and rubber tubing, scalpel, and threaded needle. Select and prepare a site, as for intravenous injection, over the jugular vein (preferably on the left side). Holding a sterilized, sharp scalpel between the thumb and index finger with only a portion of the blade exposed, make an incision from $\frac{1}{4}$ to $\frac{1}{2}$ inch long through the skin directly over and parallel to the jugular vein. Connect the free end of the tubing attached to the cannula with the inlet tube of the bleeding jar or bottle. Press the vein below the incision with the thumb until it becomes prominent, then with an inward and upward thrust insert the cannula through the incision into the vein. Continue the pressure on the vein during the bleeding. When the jar is about three-quarters full (4½ liters), stop the flow by pressing the tubing between the thumb and forefinger while the assistant disconnects the tubing from the inlet tube of the jar, and after covering the latter with a sterile cap quickly connects it to the inlet of the next jar. When the desired quantity of blood has been obtained, release the pressure over the vein. Close the incision by lateral pressure of thumb and index finger to avoid hemorrhage while withdrawing cannula. Insert a single suture and paint the site with iodine.

Allow the jars to stand undisturbed until the blood has reached room temperature and a firm clot has formed and shows separation from the wall—from three to six hours. If the clot has not separated completely, loosen it by turning the jar quickly or by cautiously tilting it; otherwise, the weight when dropped may tilt and exert uneven pressure. If the temperature of the room is low, it may be advisable to cover the jars to prevent too rapid cooling. Remove the jars to the cold room and pull the string attached to the cotterpin which releases the weight.

When blood is collected in the 2½-liter tubes, follow the same general technic. At the side of the cotton plug insert the glass tube attached to the end of the rubber bleeding tube. The weight, sterilized in a separate wrapper, is let down into the tube just before the latter is placed in the cold room.

Bleeding for Plasma.—Blood is drawn into a bottle containing 17-per-cent sodium citrate solution (*F71*) in the proportion of nine parts of blood to one of citrate. On the bottle, measure with a tape the height to which the blood should be brought and mark. Replace the stopper of the bottle with a sterile cotton plug covered with gauze. Raising the cotton plug slightly, insert a glass tube connected by rubber tubing with the cannula, then reinsert the cotton plug and press it around the tube. Proceed to bleed as for serum. Hold the bottles at room temperature for from four to six hours, then place in the cold room.

Trial Bleeding.—Proceed as above but omit the incision and use a needle (15 gauge, 1½ inches long) instead of the cannula. Unless larger quantities are specified, draw from 20 to 40 ml. of blood into a tube (180 by 25 mm.). When a trial bleeding is to be taken at the time of a large bleeding, collect the required amount of blood in the tube early in the bleeding.

Bleeding for Defibrinated Blood.—Prepare the site for bleeding. Use a needle (13 gauge, 2 inches long) or cannula (5 mm. diameter, 3 inches long) attached by tubing to a bottle or flask for defibrinated blood. When the required volume of blood, usually 200 or 300 ml., has been taken, replace the needle in the tube fastened to the bottle. Shake the bottle or flask continuously while withdrawing the blood. Continue to shake until the blood is thoroughly defibrinated.

Bleeding Out.—Anesthetize the horse after placing it in such a position that the blood can drain from the hind quarters and abdominal region. With the usual aseptic precautions, make an incision over the jugular furrow in the lower third of the neck, and by blunt dissection expose the carotid artery. Clamp or ligate the artery at two points about four inches apart. If the artery is ligated, use a knot that can be easily loosened. Then insert the cannula, directed against the blood flow, into the carotid between the clamps and ligate. Remove the clamp from the proximal portion of the artery and allow the blood to flow into the receptacle. From 20 to 35 liters of blood can usually be obtained.

Drawing Off Serum and Plasma.—Serum and plasma are drawn off under strictly aseptic precautions in a draft-free room.

The first collection of serum is usually after forty-eight hours, the second after seventy-two. Remove the bleeding jars carefully from the cold room, replace the inlet tube and stopper with a siphon, and wrap cresolized cotton about the opening, or slit the paper and insert the siphon through the weight-peg hole and wrap with cotton. To remove serum from the bleeding tubes, place a sterile receiving bottle at a lower level and insert the stopper and connections of the siphon outlet. Raise the cotton plug of the tube that contains serum sufficiently to insert the siphon, replace the plug, and press it around the siphon.

Plasma is collected only once, usually after forty-eight hours. Bring the separated blood carefully from the cold room to avoid disturbing the red cells, and remove the plasma from the bottles in a manner similar to removing serum from the bleeding tubes.

Adjust the siphon so that the inlet is below the surface of the serum or plasma, then apply vacuum to the short glass tube of the receiving bottle until the material begins to pass over. Take great care not to disturb the clot or to draw over the sedimented red cells. This is especially important in the case of serum. Immediately after use, wash the siphon in cold water. Use a separate sterile siphon and connections for each bleeding.

SHEEP

Large healthy sheep of medium-wool breeds are preferred for present laboratory requirements. Pure-bred and grade Suffolk and Corriedale sheep form the bulk of the flocks. For general information relating to sheep, see bulletins of the U. S. Department of Agriculture or publications of State agricultural colleges.

When practicable, a trial bleeding is obtained from each animal before purchase to determine whether its red blood cells would be suitable for use in complement-fixation tests.

Wide variation has occasionally been found in the resistance of the erythrocytes of different sheep to hypotonic salt solutions. In general, when a low degree of resistance has been demonstrated, the sheep's red blood cells will hemolyze during washing. Hence, the fragility test, p. 69, is used as an aid in selecting sheep and to show alterations in cellular resistance due to bleeding or other causes. Additional criteria are given in the chapter on complement-fixation tests, see, "The Hemolytic System," p. 375.

Quarantine and Special Treatment

New animals are examined, given an identification number, and quarantined for six weeks. They are dipped at least three times at from ten- to fourteen-day intervals to eradicate external parasites and again after each shearing. A 0.05-per-cent aqueous solution of nicotine sulfate held at a temperature of from 35° to 40°C. is used for this purpose. Treatments for gastrointestinal parasites are given all new sheep as soon as practicable and repeated as required.

Quarters and General Care

Quarters.—Sheep are usually divided into several small flocks and housed mainly in shelters in or adjacent to pastures or paddocks. Facilities are provided for individual and flock isolation. Stalls are available as needed for lambing pens.

Feeding of Sheep (1).—When pasture is not available or is inadequate, alfalfa hay, clover hay, or mixed hay containing timothy and from 15 to 30 per cent of clover or alfalfa is fed. Concentrates are fed once or twice daily when required. With good quality legume hay, oats and corn, alone or in combination with wheat bran, are fed frequently. With mixed hay or legume hay of poorer quality, a concentrate mixture is fed which contains, by weight, 7 parts of corn or oats or both, 2 parts linseed or soybean-oil meal, and 1 part wheat bran. Pastures are rotated, when possible, to assist in parasite control and to improve grazing. When available, grass is fed to sheep not in pasture. Water and salt are always available.

Bleedings

The procedures for bleeding are the same as for horses. Sheep are bled in a standing position in a special stock (an elevated platform with stanchion) or while an assistant holds the animal's head.

From 200 to 600 ml. of blood may be taken from a sheep every two to four weeks. An interval of four weeks between bleedings is preferred.

Occasional intervals of rest may be advisable when frequent large bleedings are taken.

Blood from sheep taken for use in complement-fixation tests is added to an equal volume of 2.5-per-cent sodium citrate solution or citrate glucose solution (*F72.5*). The blood of sheep whose red blood cells are no longer suitable for such tests may be satisfactory in the preparation of certain culture media. Defibrinated or citrated blood used for media is collected in bottles with rubber stoppers and connections similar to those described for collecting small amounts of defibrinated blood from horses, see p. 62. For citrated blood, use 1 part of a 4-per-cent sodium citrate solution for each 4 parts of blood.

FRAGILITY TEST OF ERYTHROCYTES

The degree of fragility of sheep erythrocytes may be determined by observing the amount of hemolysis occurring in hypotonic salt solutions. The fragility index is expressed by the percentage of salt solution that produces lysis of 5 per cent of the cells in twenty minutes at room temperature. It is obtained by adding a fixed quantity of cell suspension to each of a series of tubes containing sodium chloride solutions of varying degrees of hypotonicity. The percentage of lysis is determined by comparative photoelectric colorimeter readings of the supernatant in the unknown tubes and of a control representing 5 per cent of hemolysis (6–8).

Using the test described, fragility index readings of from 0.580 to 0.780 or higher have been obtained from apparently healthy sheep. Cells showing 5 per cent of hemolysis in salt concentrations of 0.700 or above have, with few exceptions, been unsuitable because of lysis during washing. Cells having a fragility index of 0.680 or less have, in general, not shown lysis during the washing process.

Hypotonic Salt Solutions

A stock solution of 10-per-cent sodium chloride is precisely prepared, and if kept properly stoppered to prevent evaporation may be used for a relatively long period. The hypotonic solutions are prepared at intervals at two months or less from the stock solution by dispensing the required amounts from a burette into 500-ml. volumetric flasks and dilution with water to the mark to give the desired concentrations. Sixteen hypotonic solutions beginning at 0.80 per cent and decreasing by from 0.02 to 0.50 per cent are usually prepared.

Erythrocytes: Blood is collected in an equal quantity of isotonic sodium citrate solution as routinely used in the collection of blood for complement-fixation tests. Twelve milliliters of a well-mixed sample are centrifuged for twenty minutes at approximately 2000 r.p.m., the supernatant is removed, and salt solution is added to make 6 ml. of a suspension of cells in a concentration equal to that of the original whole blood.

The Test

As an aid in selecting the range of hypotonic solutions to be used, a preliminary test of the citrated blood using one tube of 0.62-per-cent salt solution may be made before the original centrifuging.

Set up eight stoppered tubes; one containing 9.5 ml. of 0.85-per-cent salt solution, the others the same amounts of chosen hypotonic solutions. To each add 0.5 ml. of the cell suspension thoroughly mixed just previously by repeated inversion of the containing tube. It is important that the contents of each tube be mixed immediately after addition of the cell suspension. Allow to stand twenty minutes at room temperature, centrifuge without delay, and remove the supernatant for comparison with a standard hemoglobin solution representing complete hemolysis of a 1:400 dilution of the cell suspension. The standard is prepared by dilution of 0.5 ml. of the original cell suspension with water to 200 ml. in a volumetric flaks.

Readings

With a No. 54 green filter, in a Klett-Summerson photoelectric colorimeter, set the instrument at zero for distilled water and make a reading (R_S) with the standard hemoglobin solution. Readjust to zero for the supernatant from the tube with 0.85-per-cent salt solution, which should be free from hemoglobin, and make readings (R) with the several hypotonic supernatants. The lysis expressed in per cent (H) may be obtained by dividing the reading of the unknown by two-tenths of the reading of the standard. This accounts for dilution of the cell suspension 1:20 in hypotonic solutions and 1:400 in the hemoglobin standard. It is convenient to employ a series of hypotonic salt concentrations (X) at intervals of 0.02 per cent. When the resulting values of H (degree of hemolysis) are plotted on coordinate paper as ordinate against salt concentration X in descending order of magnitude, the points describe an ascending curve with gradually increasing slope; that is, as X decreases, H increases. If, however, the logarithms of the lysis percentages are plotted instead, the described curve appears approximately straight within the range from 2- to 12-per-cent hemolysis. Accordingly, this furnishes a basis for linear interpolation to estimate precisely the percentage of salt required (X_F) to produce 5-per-cent hemolysis under the given conditions.

The simplest procedure in obtaining X_F is to use the values of log R from the colorimeter readings, that for the standard being log R_S.

Then:

$$X_F = X_1 - (X_1 - X_2)\,\frac{\log R_s - \log R_1}{\log R_2 - \log R_1}$$

where $R_1 \leqq R_S \leqq R_2$; i.e., values of R such that R_S lies between them.

Example:

Per cent NaCl (X)..	0.68	0.66	0.64	0.62	0.60	0.58	0.56	Hb standard R_S
Colorimeter reading (R)	22	40	79	148	274	510	810	96
log R........	1.342	1.602	1.898	2.170	2.438	2.708	2.909	1.982
Per cent lysis (H)........	1.15	2.08	4.11	7.72	14.3	26.5	42.1	

$$X_F = 0.64 - (0.64 - 0.62)\frac{1.982 - 1.898}{2.170 - 1.898} = 0.6338$$

Similarly:

$$X_F = 0.66 - (0.66 - 0.60)\frac{1.982 - 1.602}{2.438 - 1.602} = 0.6327$$

$$\text{Averaging } \frac{0.6338 + 0.6327}{2} = 0.633$$

$$X_F \text{ (Fragility Index)} = 0.633$$

GOATS

Since goats at the present time are used mainly for intracutaneous tests, animals with light-colored skins are required. Those of the Saanen breed have been found particularly suitable. The general management of goats is similar to that of sheep. When a goat is received, its blood is tested for agglutination reactions with *Brucella melitensis* or *Brucella abortus* in order to guard against possible introduction of carriers into the herd.

The method for the intracutaneous injection of goats is similar to that for rabbits. Remove the hair from the back and sides by close clipping and protect the animal from cold and direct sun rays.

The procedures for the post-mortem examination of sheep and goats are similar to those for other large animals (4).

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CHAPTER 5

USE OF EXPERIMENTAL AND TEST ANIMALS

The operative procedures commonly used in laboratory work that requires animals are those of injection, test feeding, bleeding, and postmortem examination (1–6).

During and following all operations upon living animals the utmost consideration of the animals must be observed. Rules regarding treatment of animals are posted in the operating rooms.

Adequately equipped operating rooms and draft-free rooms for procedures requiring special precautions adjoin certain of the test animal quarters; in some, benches and equipment are available for operative procedures. Tests in animals of material that is extremely infective or is suspected of containing pathogenic sporeforming microorganisms are made in rooms assigned for such purposes.

IDENTIFICATION OF ANIMALS

Rabbits are identified by a number tattooed on the inner surface of the right ear before they leave the breeding quarters or on receipt from outside sources. Guinea pigs are identified by a numbered aluminum disk attached to the ear immediately before use; mice, by stains applied to the tail and body; monkeys, by a number tattooed on the chest.

OPERATIVE PROCEDURES

ANESTHETIZATION (7)

Masks of different sizes are used for anesthetizing rabbits and guinea pigs. The mask is provided with an open window for the insertion of gauze—one layer for guinea pigs, two for rabbits. Ether is dropped on the gauze until the surface is moist, then continued more slowly until the operation is completed. The depth of the anesthesia is controlled by the rate of administration. Too much ether is indicated by extremely rapid, shallow breathing, irregular breathing, or spasms which usually appear first in the hind legs and are synchronized rhythmically with the breathing. Insufficient ether is indicated by respiratory spasm and struggling. When administering an anesthetic to an animal which is to be bled, decrease the rate of administration as the blood is removed and discontinue when about half of the volume has been collected. After use, discard the gauze and clean or sterilize the mask.

General anesthesia is usually preferred but local anesthesia, as by means of from one-half- to two-per-cent procaine hydrochloride, may be used if indicated. A convenient apparatus for anesthetizing mice is illustrated in figure 7. A bottle of ether is set in a battery jar containing water warmed to about 35°C. Air is bubbled through the ether and then introduced into the bottom of a second battery jar through a metal tube. The second jar is fitted with a wire-grid raised from the bottom on which the animals are placed. The amount of ether can be gauged by the rate of bubbling and controlled so that mice are anesthetized in about twenty or thirty seconds. Mice are usually put in the jar one by one at about five- or ten-second intervals or as rapidly as injections can be made.

INJECTION

Injections or inoculations are usually made by the intracutaneous, subcutaneous, intravenous, intraperitoneal, or intracerebral method. Sterile instruments and aseptic technic are required. One- or two-per-cent cresol compound, 70-per-cent alcohol, and tincture of iodine are used singly or in combination for disinfection of the skin.

Preparation of the Animal.—Use one of the following methods when hair is to be removed from the area at the site of injection:

1. Clip with an electric clipper equipped with close-cutting surgical blades.
2. Clip with hand clipper and shave.
3. Clip and depilate with equal parts of barium sulfide and cornstarch mixed with water to make a paste. Apply the paste, allow it to remain for three or four minutes, then wash off thoroughly with lukewarm water. The animal should be kept warm until it is dry.

Operating trays for guinea pigs and rabbits: Trays of Monel metal, stainless steel, or zinc are constructed with a central longitudinal depression 1½ inches deep and with studs and cleats at each end for securing ties. Three sizes are used, 10 by 18 inches for guinea pigs, 12 by 26 inches for small or medium-sized rabbits, and 12 by 32 inches for large rabbits. Similar trays made of masonite have been found satisfactory.

Preparation of Syringe. Use a glass syringe and a hypodermic needle that have been sterilized by hot air, steam under pressure, or boiling water. For the first two methods, sterilize the needle, barrel, and plunger separately in tubes or wrap the barrel and plunger separately in paper. For the third method, separate the needle, barrel, and plunger and sterilize by boiling in water for five minutes. Allow time for the needle and syringe to cool before use.

When necessary to avoid even slight dilution of the test material, first rinse the syringe and needle by drawing up and expelling a small amount of the material to be injected. When blood cells are to be given, use a dry syringe or one previously rinsed with sterile salt solution.

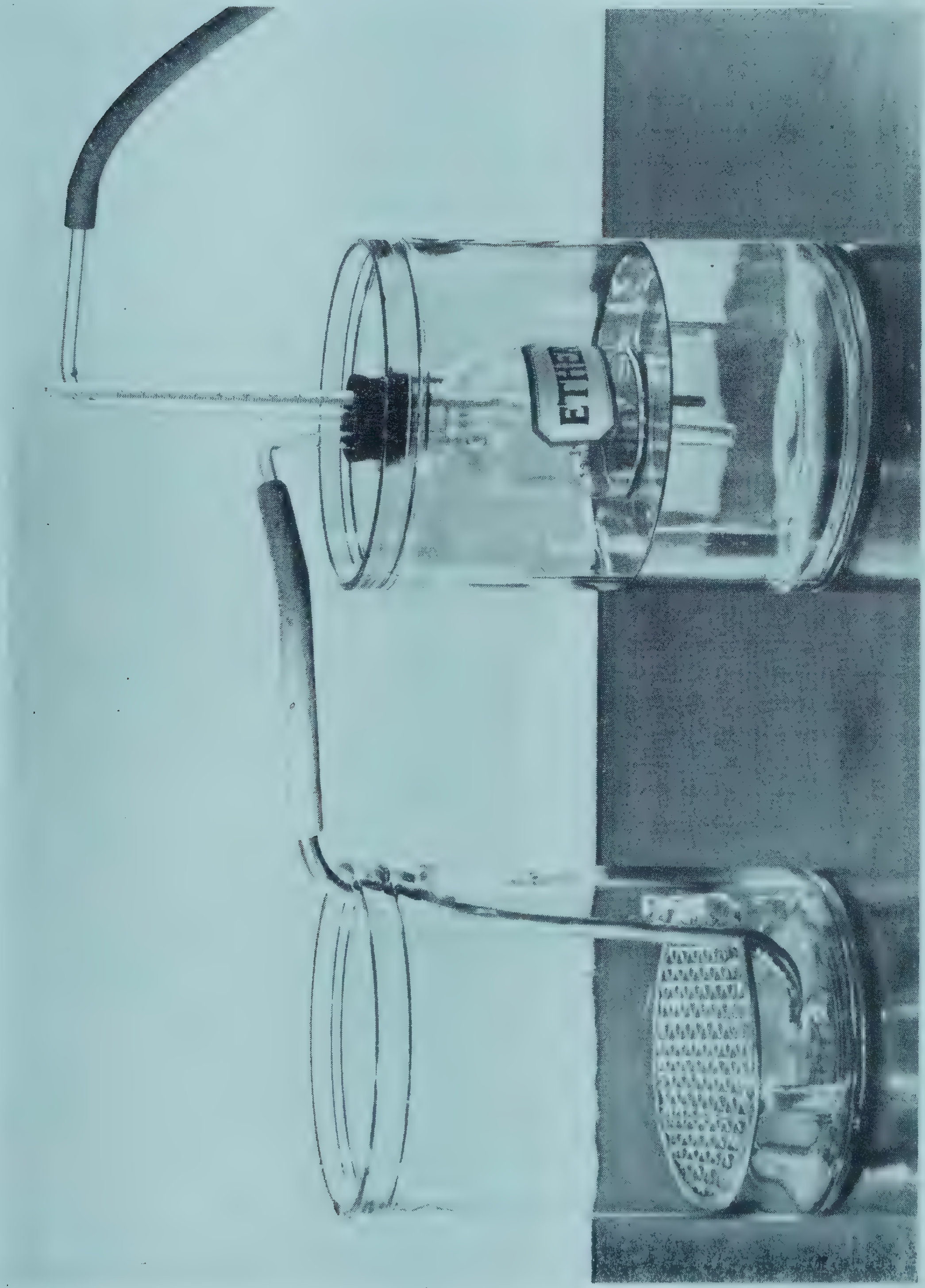

Fig. 7. Apparatus for Anesthetization of Mice

In filling the syringe, draw into the barrel a little more material than is necessary for the injection. Invert the syringe and force out any air that may have entered. If the material is infective, either cover the lumen of the needle with cotton moistened with 1-per-cent cresol or with alcohol or previous to filling, remove air from syringe by replacement with sterile saline or water.

Rabbits

To lift a rabbit, move one hand forward over its back and gently grasp the ears and a fold of skin over the shoulders. The weight of the animal should be mainly supported by placing the other hand under the posterior quarters.

For intracutaneous injection, use a 26-gauge needle, one-quarter or three-eighths of an inch long. Have the rabbit held or tie it to a tray. It should be held securely, but the skin should not be stretched taut at the point of injection. With the bevel of the point up, introduce the needle between the layers of the skin, taking care that it does not go through to the subcutaneous tissue. A bleb should form as the material is being injected.

For subcutaneous injection, use a needle from 24- to 19-gauge, from three-quarters to one and one-half inches long. Restrain the animal and introduce the material under the skin. If the injection is made over the abdomen, take care to avoid puncturing the peritoneal wall. To facilitate the procedure, raise the skin slightly just in front of the point of insertion.

When introducing solid material that cannot be emulsified, proceed as follows: With the animal under general or local anesthesia and using sterilized instruments, snip the skin with sharp-pointed scissors to form a V-shaped opening about 1 cm. in length. Lift up the lower edge of the opening with forceps and force the closed blades of scissors under the skin. Open the blades to form a pocket and then close and withdraw them. Insert the inoculum into the pocket, close it with a single suture, and disinfect the surface of the wound with alcohol. When very little material is inoculated and the opening is small, close it with collodion.

For intraperitoneal injection, use a needle from 24- to 19-gauge, from three-quarters to one and one-half inches long. Restrain the animal and introduce the needle subcutaneously with the point toward the head, anterior to the region of the groin, then slant the point of the needle downward and pierce through the peritoneal wall.

For intravenous injection, use a needle from 26- to 22-gauge, from three-eighths to one and one-half inches long. Place the rabbit in a special box or have it held by an assistant.

If the latter method is to be used, place the rabbit on the bench and grasp it under the abdomen in such a way that the hind legs are held firmly between the thumb and second finger while the index finger rests against the inner side of the right hind leg. Grasp the forelegs in a similar manner, lift the animal from the bench, and stretch it at full length. In the case of some animals, no restraint is necessary.

The marginal ear vein is most accessible. Rub the site of injection lightly with cotton moistened with 70-per-cent alcohol until the veins become prominent. Rest the ear against the first three fingers of the left hand and hold it down with the thumb. After making certain that no air remains in the syringe, introduce the needle, directed toward the head, into the marginal vein. Inject a very small amount of the material. If a bleb forms, indicating that the needle is not in the vein, withdraw the needle and insert it again nearer the base of the ear. When the needle is in the ear vein, make the injection slowly. Remove the needle and if there is bleeding place a small piece of dry cotton over the point of injection.

When several injections are to be made into the same vein, begin toward the tip of the ear so that there will be space for later injections.

Guinea Pigs

Lift guinea pigs with the hand grasping the sides of the animal lightly under the forelegs. When carrying, support them with the hand or arm.

For intracutaneous injection, hold the guinea pig in an extended position on its side with one hand grasping the head and forelegs, the other the hind legs. (See Fig. 8.)

For subcutaneous or intraperitoneal injection, place the left hand around the neck of the guinea pig so that the right paw is held firmly between the thumb and first finger, the left between the first two fingers. Invert the animal and place the right hand underneath so that the thumb and first finger hold the right hind leg, the first and second, the left. Then stretch the guinea pig across the knee in position for injection. For injection in the groin, see Fig. 9.

For intravenous injection, use a 26-gauge needle, one-quarter or three-eighths of an inch long. Tie the animal securely to a tray, with the ventral surface down, and shave the right hind leg. Cleanse with 70-per-cent alcohol or 2-per-cent cresol compound, and slip a support, such as a 12-mm. tube, between the leg and the tray. Apply pressure across the upper part of the leg or distend the vein by placing an elastic band around the leg before the area is cleansed. Following general or local anesthesia, make a short incision, from 0.5 to 1 cm., diagonally from the upper and inner side of the leg and expose the saphenous vein. Insert the needle well into the vein, cut the band, and inject the material slowly. Remove the needle, wipe the area with 70-per-cent alcohol, and if necessary to stop bleeding press a piece of cotton over the incision.

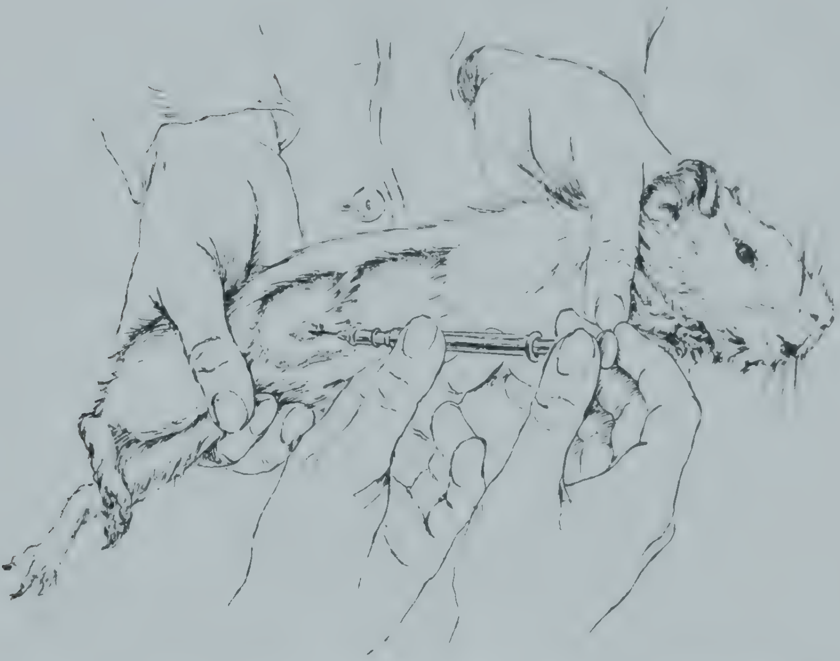

FIG. 8. INTRACUTANEOUS INJECTION OF A GUINEA PIG

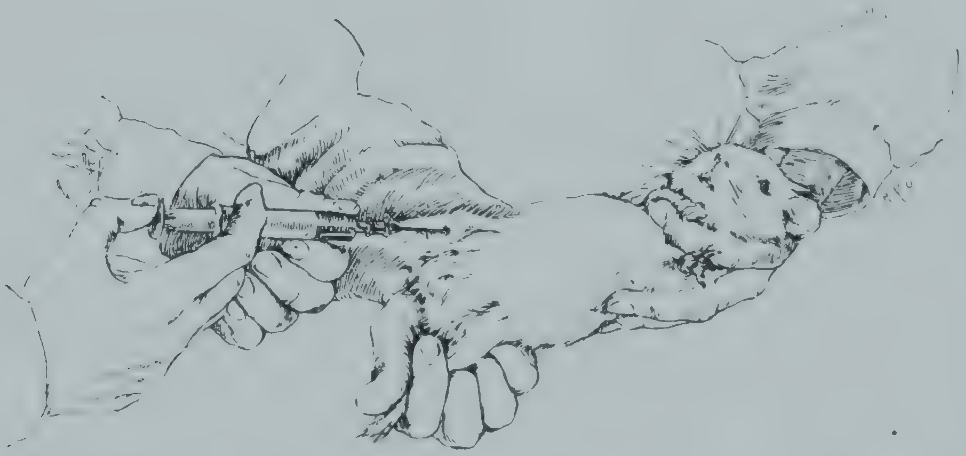

FIG. 9. SUBCUTANEOUS INJECTION IN THE GROIN OF A GUINEA PIG

Mice

For subcutaneous or intraperitoneal injection, hold the mouse by the tail with the right hand and allow it to stretch full length. With the thumb and index finger of the left hand grasp the skin at the back of the neck quickly and firmly. Turn the hand palm up and keeping the mouse stretched out, secure the tail and left hind leg against the base of the thumb with the third or fourth finger. (See Figs. 10 and 11.)

For intravenous injection the tail vein is generally used. Just before the injection dip the tail into warm water (50°C.) for from one to two minutes. Place the mouse in a short metal cylinder about 1½ inches in diameter and open at both ends, or have an assistant hold the mouse. Grasp the tail with the left hand, introduce the needle (27 gauge, five-eighths of an inch long, short bevel) into one of the distended lateral veins, and inject the material slowly. If several injections are to be made on the same animal, begin toward the end of the tail and make each succeeding injection nearer the body.

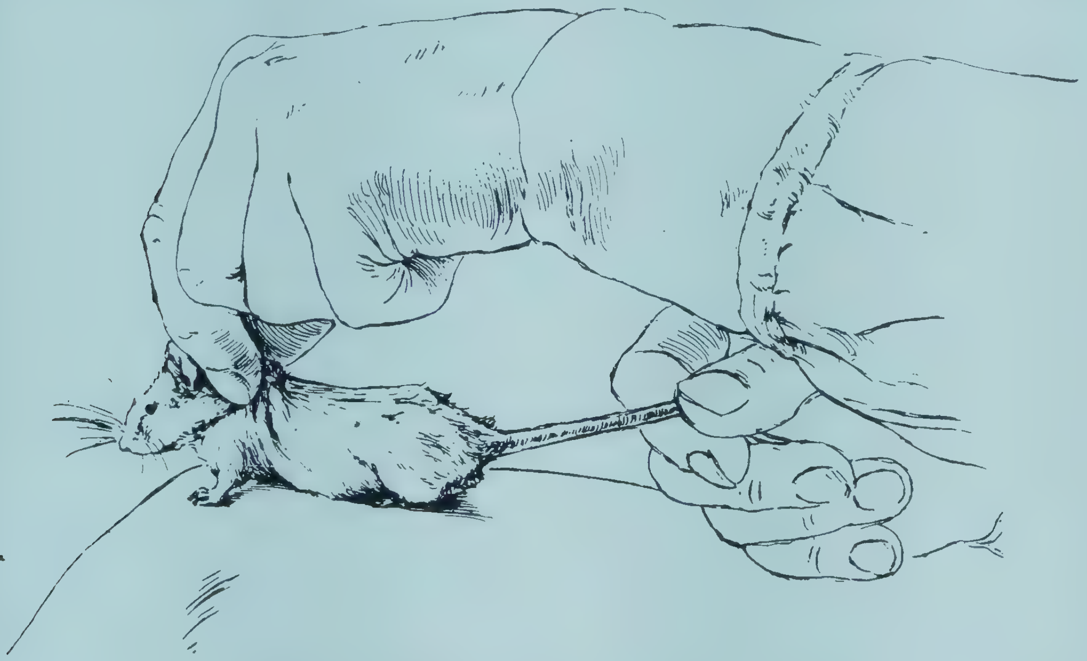

Fig. 10. Manner of Grasping a Mouse

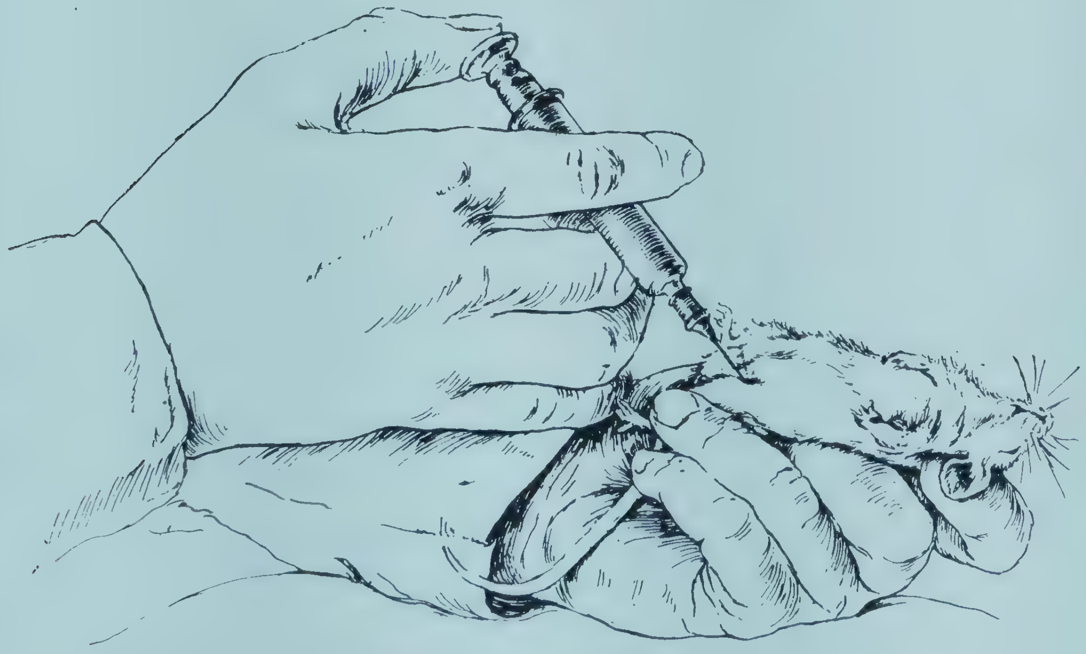

Fig. 11. Intraperitoneal Injection of a Mouse

For intracranial injection of mice, etherize lightly, and wet the skin over the skull with alcohol. Inject intracerebrally from 0.02 to 0.04 ml. of the test material with a small syringe and a $\frac{3}{8}$ inch, 26-gauge needle. Introduce the needle through the skull at a point slightly to the left or right of the mid-line and just anterior to a line drawn from ear to ear. Intracranial injection of a guinea pig or rabbit is made similarly after trephining the skull while the animal is under general anesthesia.

Rats

The methods for injecting rats are the same as those used for guinea pigs. To restrain the rat, grasp it by the tail with the right hand and at the back of the neck with the left hand protected by a leather glove. Turn the animal and hold it securely in the position desired for subcutaneous or intraperitoneal injection. Intravenous injections may be made into the femoral or saphenous veins following general or local anesthesia or into one of the caudal veins without anesthesia.

Monkeys (Macacus rhesus)

Use a net or allow the monkey to leave its cage and climb on the wire front or on that of an adjacent cage. Grasp it across the small of the back with the right hand and press its body firmly against the cage or wall to prevent its turning, then grasp it around the neck with the left hand. With the right, hold the monkey's arms behind its back, then release the neck. The animal may be carried safely with one hand. When the monkey is to be anesthetized or injected, grasp the legs with the left hand and stretch out the animal.

The methods for intracutaneous, subcutaneous, and intraperitoneal injections are similar to those for the rabbit.

Make intravenous injections into the median basilic vein at the bend of the elbow, applying a tourniquet until the needle enters the vein. To give intracerebral injections, anesthetize with ether and insert a needle (20 gauge, $\frac{5}{8}$ inch long) through a small trephine hole in the skull $\frac{1}{2}$ to 1 cm. to one side of the midline on a line connecting the external auditory meatus. For intranasal inoculation, close one nostril with cotton and insert the soft rubber tip of a small "Asepto" syringe into the other, gently expelling the contents.

TEST FEEDING

Draw the emulsified material to be fed into a 5-ml. syringe or into the larger end of a 5-ml. pipette and attach a piece of soft rubber tubing (three inches long for guinea pigs and rabbits). Wrap the animal in a towel to prevent struggling. Open the animal's mouth with forceps or by pressing a finger against the jaws and insert the tubing. Allow the fluid to flow gradually to avoid choking.

The test material may be mixed with the animal's food, the usual procedure for feeding mice, in which case the feeding immediately before may be withheld, or to encourage prompt consumption a more appetizing food may be mixed with the test material.

When feeding by stomach tube an oval mouth speculum may be used to introduce the tube. As the animal swallows, push the tube very gently down the throat.

BLEEDING

In order to obtain a clear serum, bleedings are usually taken in the morning following an overnight period of fasting. If a considerable quantity of blood is taken, water should be provided immediately afterwards.

Rabbits

From the Ear Vein.—Place the rabbit in a bleeding box. Close the box carefully to avoid injuring the animal, then turn it on end so that the animal's head is down. (See fig. 12.) When the temperature of the room is low or when a steady flow of blood cannot be obtained, better results may be had by filling the jacket with warm water. Small amounts of blood may be taken from some rabbits without the use of the box.

Remove the hair from a small area over the marginal vein and along the edge and lower surface of the ear. Stimulate the circulation and disinfect the ear by rubbing it lightly with cotton moistened with 70-per-cent alcohol, and make a short longitudinal incision. When sufficient blood has been obtained, press a small piece of dry cotton against the opening until a clot has formed.

Bleeding-Boxes for Rabbits.—Boxes are made of rust-resisting metal and provided with double walls that form a jacket for water, adjustable cover with head opening, and end piece. Two sizes are available, one for small or medium-sized rabbits, the other for larger rabbits. Similar boxes made of wood and masonite without water jacket are frequently preferred because of lightness and ease in handling.

From the Heart.—Tie the animal securely on a tray. Prepare an area, 1½ inches or more in diameter, on the chest over the point of maximum pulsation of the heart. Introduce the needle (19 gauge, 1½ or 2 inches long) at this point. Before the needle enters the heart, apply suction by mouth or a low degree of vacuum by means of a pump and a piece of rubber tubing previously attached to the outlet tube of the bottle in which the blood is to be collected. Continue the vacuum as necessary until sufficient blood has been obtained, then carefully withdraw the needle from the heart. Close the inlet tube by a clamp or otherwise and withdraw the needle from the chest wall. When a small amount of blood, from 10 to 20 ml., is required, collect it in a glass syringe. If the blood is not forced into the syringe by the heart action, draw up the plunger very gradually. Remove the needle and empty the contents of the syringe immediately into a sterile container.

From 20 to 40 ml. of blood may be taken from a rabbit that weighs from 2000 to 3000 grams at intervals of two or three weeks.

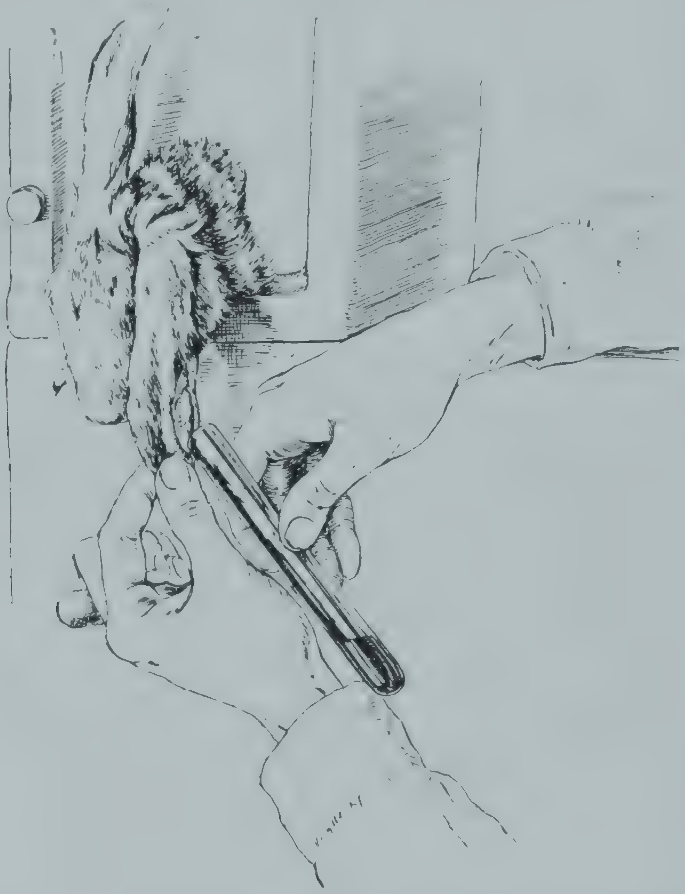

FIG. 12. BLEEDING A RABBIT FROM THE EAR VEIN

From the Carotid Artery.—Anesthetize the animal on an operating tray tilted slightly so that the head is low. Prepare the site of operation and make a longitudinal incision through the skin. Lay back the skin on either side and with forceps and scissors cut down very carefully until the carotid artery is exposed and freed from surrounding tissues. Apply artery clamps and carefully raise the artery with forceps. Make a very small incision with fine, sharp scissors and insert the tip of a tenaculum. Then insert the glass cannula attached to the bottle in which the blood is to be collected. Hold or tie the artery firmly on the cannula and without pulling the artery release the upper clamp and allow the blood to flow into the bottle. After the flow has ceased, remove the cannula from the vein, and seal the tip in a flame. Exsanguination by bleeding from the heart has largely replaced this procedure. A light ether anesthesia is used.

Guinea Pigs

From the Heart.—Follow the procedure described for bleeding rabbits but use a syringe.

From 10 to 15 ml. of blood may be taken from a guinea pig weighing 700 grams at intervals of two or three weeks.

From the Carotid Artery (for Complement).—Anesthetize or stun the animal by a quick blow at the base of the skull. Expose the trachea and blood vessels by removing a section of the skin. Dissect and cut the carotid artery or cut transversely the blood vessels on either side of the trachea. Hold the guinea pig with the head bent backward at a right angle and allow the blood to collect in sterile Petri plates.

Monkeys

Blood may be obtained by intracardiac puncture following the technic described for rabbits. To obtain small amounts, the median basilic vein is selected. Place a tourniquet on the arm above the site of operation. Use a syringe with a 26-gauge needle, from one-half to three-quarters of an inch long. When the needle is in the vein, loosen the tourniquet and withdraw the blood slowly.

Cerebrospinal fluid: Spinal fluid is best obtained by cisternal tap. Use a special needle (20 gauge, 1 inch long) with a second hole filed in the back of the shaft at the level of the upper end of the bevel and provided with a wire or stylet which does not project beyond the point. Anesthetize the monkey and place it face downward on the table with its head flexed over the edge. Shave the site of puncture at the base of the skull and paint with iodine. Insert the needle with its stylet still in place through the skin so that the point strikes the skull and slides downward passing through the posterior occipitoatlantal ligament. Insert the needle until the hub is flush with the skin and then withdraw the stylet. Draw into a capillary pipette the fluid which wells up in the hub of the needle and transfer it to a sterile tube.

Drawing Off Serum

Place the tube or bottle that contains the blood in a slanting position until the clot has shrunk. Rim the clot if necessary with a 0.2-ml. pipette and store in the cold room. The next day flame the top of the tube or bottle and pour or pipette the serum into a centrifuge tube. Centrifuge to remove the blood cells and pipette or pour the serum into a bottle.

OBSERVATION AND CARE OF ANIMALS UNDER TREATMENT

WEIGHT AND TEMPERATURE DETERMINATIONS

When a record of the weight of an animal under observation is required, weigh it on the day of the test and on each succeeding day at approximately the same hour, preferably in the morning before feeding.

Use a veterinary or a human rectal thermometer. Lubricate and insert it to a uniform distance in the rectum of the animal. For routine determinations of the temperatures of guinea pigs the insertion should be from 2½ to 3 cm.; to insure greater accuracy, from 5 to 6 cm. may be necessary.

The temperature of a normal animal may vary considerably and be influenced markedly by such factors as excitement or activity. The average temperature of guinea pigs has been found to be 101.7°F. at a thermometer insertion of 3 cm., 102.2°F. at 6 cm.; that of the rabbit 103.3°F. at 6 cm.

POST-MORTEM EXAMINATIONS

A general knowledge of the anatomy of laboratory animals may be obtained from textbooks (1–4). For data on their diseases, see "Care and Breeding of Laboratory Animals," p. 51.

Post-mortem examination of test animals should be made as soon after death as practicable. When delayed, the animal should be transferred promptly to a low-temperature chamber.

Sterilize the required instruments in boiling water for at least five minutes. When cultures are to be taken, use fresh, sterile instruments for each step in the procedure. Tie the animal to an operating tray, or if it is a mouse fasten it to a board with push pins. Moisten the hair over the thorax and abdomen with 1-per-cent cresol, or when highly infective material is being studied, dip the animal in the cresol solution.

Make a primary incision through the skin from the symphysis pubis to the base of the neck, then transverse incisions in the region of the posterior third of the abdomen and over the thorax. Loosen and turn back the skin, exposing the ventral wall. Note the condition of the abdominal wall at the site of inoculation and of the inguinal and axillary lymph nodes. Take cultures and make slide preparations from the lesions as indicated, the choice of media being determined by the kind of material injected and the nature of the lesion.

Make a midline incision through the peritoneal wall. Note the amount and character of the peritoneal fluid and take cultures and make slide preparations if indicated. Fold back the peritoneal wall after making transverse incisions along the margin of the ribs and at the posterior end of the long incision for examination of the abdominal viscera.

Open the thoracic cavity from base to apex by cutting costal cartilages on both sides. Connect the two incisions by cutting through the diaphragm. Lay back the sternum; note the quantity and appearance of the pleural fluid and take cultures and make slide preparations if indicated. Expose the heart by opening the pericardial sac and note the amount and character of fluid. If blood is to be taken for culture, raise the heart with hemostatic forceps clamped above the auricles. Sear lightly and remove the apex with scissors or make an incision through the wall of the right ventricle. (See Fig. 36, p. 273.)

The extent and character of the examination vary with the purpose for which the test was undertaken. The particular procedure to be followed in each instance is indicated in the specific chapter. In order to disclose possible sources of infection among normal stock, the following minimum routine procedures have been adopted in the post-mortem examination of animals dying without gross lesions. These procedures are based on the results of a large number of routine necropsies of uninoculated animals. Rabbits: inoculate a blood-agar plate (*F43A*) with material from the lungs. Guinea pigs: inoculate blood agar and Endo agar (*F52A*) respectively from the lungs and spleen. Mice: inoculate Endo medium with material from the spleen.

Tissue for Histologic Examination.—When a histologic examination of tissue is necessary, proceed as soon as possible with the necropsy. Place representative sections of the tissues to be examined, not over 0.5 cm. in thickness, in Zenker's fluid and in 10-per-cent formalin. Use at least from 10 to 20 parts of fixative to one of tissue. Refer gross specimens that are of interest directly to the pathologic laboratories for preparation for the museum, or place them first in the proper fixative.

PROCEDURE IN TESTS REQUIRING SPECIAL PRECAUTIONS

Special precautions against the spread of infection are required in the case of animals inoculated with material known to contain or suspected of containing *Bacillus anthracis*, *Bacterium mallei*, *Bacterium tularense*, *Pasteurella pestis*, *Vibrio cholerae*, microorganisms of the abortus-melitensis group, and certain other species of microorganisms or viruses that may incite extensive outbreaks among laboratory animals. Such animals are kept in isolation cages with deep trays, metal boxes, or glass jars in rooms reserved for the purpose. If the infection can be spread by insects, use screened boxes or jars set in shallow trays containing water and sufficient kerosene to form a film on the surface. When handling such animals, wear rubber gloves sterilized after use by 5-per-cent cresol compound. For other precautions in handling infective material, see "General Bacteriologic Technic," p. 4.

After necropsy, wrap the animal in several thicknesses of heavy paper, together with the wooden board if used as an autopsy tray, and place in an incinerator. Also, place the boxes or jars occupied by the animals, together with the bedding, water bottles, etc., directly in the autoclave.

Animals that survive the tests are chloroformed and disposed of as above.

DISCHARGE OF ANIMALS

Guinea pigs and rabbits on completion of tests are sent, if suitable for other purposes, to the "used animal" quarters with a record of the date and nature of the test and the date of discharge. Most of the guinea pigs after eight weeks are redistributed to be bled for complement. Surviving mice and rats are usually chloroformed.

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CHAPTER 6

MAINTENANCE OF THE COLLECTION OF TYPE CULTURES

The collection of type cultures consists of selected representative strains of pathogenic and nonpathogenic microorganisms. It contains a relatively large number of strains of pathogenic bacteria, selected species of fungi, and a few protozoa and viruses.

The collection is maintained to provide this laboratory and the local approved laboratories in the state[1] with cultures for use as standards in various diagnostic procedures and for the production of vaccines and sera. The maintenance of the collection and the detailed periodic examination of each strain provide materials and opportunity for the technical training of students and new members of the staff in systematic bacteriology, and for the investigation of problems related to the identification and classification of microorganisms. The possibilities for morphologic study are notably extended by the installation of an electron microscope (RCA type EMU). The differentiation of closely related microorganisms is completed by members of the staff who have specialized in the particular fields. Advances in knowledge of the biology and relationships of the various species are incorporated in the procedures for identification as their practical value is demonstrated. In the absence of international agreement regarding nomenclature, a conservative policy is followed, that is, the newer terminology is adopted as it comes into general use not only in this country but also abroad.

The cultures of the collection are divided into two groups. The representative strains, including those used as standards in the Diagnostic Laboratories and in the Antitoxin, Serum, and Vaccine Laboratories, form the first and relatively permanent division. All of the strains in this group are maintained in the collection; the majority are also maintained in the departments in which they are in active use. The second group is comprised of supplementary strains, and includes both identified and unidentified cultures under special investigation; they are maintained in the laboratories in which they are used. In order to centralize the records of available cultures, all strains are, however, first referred to the collection where accession entries are made. When a special study is completed, strains of particular interest are returned to the collection for storage. Strains unsuitable for maintenance are discarded after pertinent entries have been made on the history card and in the accession book.

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[1] The Commissioner of Health is authorized by the Public Health Law of the State (Article II §4-b) to issue to laboratories certificates of approval covering such laboratory examinations as the State Sanitary Code may require. The Public Health Law also requires that all laboratories in the state (exclusive of New York City) where live pathogenic microorganisms or viruses, other than vaccine virus, are handled or cultivated shall be registered with the State Department of Health (Public Health Law, Article XXIII, §451). This registration does not apply to laboratories maintained by the federal government, the state, a municipality, or a county.

Citations from the Law and the Code are given in the appendix.

RECEIPT OF CULTURES

Assign an accession number[2] to each culture as soon as it is received. Record the number in the accession book and on the history card, together with the date, the source and identity of the culture, and the department that is to be responsible for its maintenance. On the back of the history card record all significant data concerning the culture. Deliver new cultures that have been secured for special investigations to the member of the staff who requested them; he should report the results of his examination to the collection group promptly for the official record. Make the required examinations of all cultures immediately and record a summary of the results on the front of the history card. If a contaminated culture is received, retain a fishing with characters typical of the species and note the fact on the history card.

If the results of the tests indicate that the culture is unsatisfactory for maintenance in the collection, notify the members of the staff who are concerned and, unless otherwise directed, discard it. Enter "Discarded" and the date in the accession book; make similar entries with a brief explanation on the history card and file under "Cultures discarded."

TESTS OF PURITY AND IDENTIFICATION

Each new culture is examined for purity and for characteristic biologic activities at the time of its receipt and after storage for one year. Cultures of the permanent collection are re-examined at least every five years; those used for the preparation of vaccines and diagnostic and therapeutic sera, at more frequent intervals. (For details of these latter tests see the sections that describe the preparation of vaccines and sera.) All strains in which contamination is suspected are immediately studied.

The procedures for the tests of purity and identification vary with the species and depend to some degree upon the purpose for which the culture is used. For example, in the examination of cultures that serve as controls in the various diagnostic procedures, the methods used in the Diagnostic Laboratories for the isolation and identification of the particular species are applied. The following outline indicates the general procedures for microorganisms that grow readily in the usual media; the technic is that described under "General Bacteriologic Technic," p. 3.

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[2] Cultures recorded in the collection before January 1, 1932 were numbered in the order of their receipt. Those recorded since have been numbered in a similar manner, except that the number is preceded by the last two digits of the year—that is, the first culture received in 1937, 3701, the tenth, 3710; the first in 1938, 3801, etc.

I. Original culture
  A. Make a film and stain by Gram's method

     If the culture is dried, prepare the film from the first subculture.

  B. Inoculate media for
    1. Preparation of dried cultures for storage
    2. Other method of maintenance, as indicated

       This transplant is usually necessary only when a new strain is received dried or when the practicability of storage in the dry state has not been established for the species.

    3. Examination for purity and identity
       As soon as growth is obtained in the subcultures cover the original culture or, in the case of dried cultures, a transplant with sterile mineral oil (U.S.P. light) to a depth of at least 2 cm. (1, 2). Store these cultures for one year at 4°–6°C. or at other suitable temperature in a basket labeled "Original Cultures." Test for viability in six months or after a shorter period, depending upon the species. If growth is not obtained, determine the viability of the dried preparations. Store transplants under oil for the remainder of the year.

II. Examination for purity
  A. Examine morphologic characters in the film from the original culture (I A).

     Keep this stained preparation for reference for one year in the case of new strains; otherwise, until the examination of the strain is completed.

  B. Study appearance of isolated colonies on plates

     Examine inoculated plates of solid medium after incubation for one, two, and seven days. With anaerobic microorganisms, examine a duplicate set of plates incubated under anaerobic conditions after two, four, and seven days. If different kinds of colonies develop, compare the characters of fishings from isolated colonies with those of the seed culture. Should contamination be found in a stock strain, examine older stored cultures and select for maintenance one that is pure and typical.

III. Study of biologic activities of pure culture

  Save transplant (I B 3) until examination is completed.

A. Morphologic characters
  1. Films from original culture and subculture in broth
  2. Motility in broth
  3. Additional microscopic preparations stained by special methods: acid-fast, capsule, flagella, spore, etc.

B. Cultural characters
  1. Colonies on solid medium
  2. Appearance of growth in broth
  3. Requirements for growth—temperature, oxygen, etc.
  4. Formation and solubility of pigment

C. Biochemical activities
  1. Hemolysis of red blood cells
  2. Liquefaction of coagulated serum
  3. Liquefaction of gelatin
  4. Digestion of meat
  5. Production of indole
  6. Fermentation and peptonization of milk
  7. Fermentation of carbohydrates
  8. Methyl red test
  9. Formation of acetylmethylcarbinol (Voges-Proskauer reaction)
  10. Utilization of citrate

D. Pathogenicity

Determine virulence and toxigenicity whenever they are of special significance in the identification of the microorganism.

E. Antigen-antibody reactions

Test for agglutination or precipitation all strains of species for which sera are available.

F. Additional examinations

Make further studies as required, so that essential data may be available regarding the biologic characters: thermal death-time and limits of growth, antigenic activities, etc. The use of single-cell methods of isolation and study may be necessary to determine individual variation.

PRESERVATION OF CULTURES

Studies of the preservation of bacterial cultures by drying have yielded such satisfactory results that many species of the collection are stored in the dry state only. For this purpose a modification of the method described by Swift (3) is in general use; the lyophile process (4, 5) is also utilized, to a limited extent. Other cultures are maintained in beef-infusion gelatin, in special media, or by passage through animals. Preservation of cultures by storage in a dry-ice box (6, 7) is under investigation, chiefly for those species for which desiccation has proved of limited practical value. Tables 3 and 4 indicate the methods used for the maintenance of the different microorganisms.

Storage in the Dry State. Grow the cultures on a favorable medium for from sixteen to twenty-four hours or longer if the microorganism grows slowly. Prepare heavy suspensions of cultures grown on solid media by removing the growth in a small quantity of broth (usually *F5A*, 0.5 to 1.5 ml. per slant in 13- by 166-mm. tube). Concentrate broth cultures by discarding nine-tenths or more of the supernatant after centrifugation.

With some species, such as pneumococci, all of the fluid is discarded and the bacterial cells are resuspended in fresh broth to which 5-per-cent normal horse or rabbit blood has been added.

With a pipette, dispense 0.1 ml. of the concentrated suspension in each of five or more tubes (11- by 75-mm.) that have been labeled with the culture number and date by means of glass marking ink covered with a label varnish. Before discarding the pipette, place a very small drop of the suspension on a plate of blood agar (*F43A*); streak it over the surface of the medium with a loop. Prepare a film, stain by Gram's method, and examine for contaminants.

Trim the cotton plugs and push them slightly below the tops of the small tubes to facilitate rapid sealing after the suspensions have been dried. Freeze the culture suspensions rapidly by standing the tubes in an upright position on a piece of dry ice with the bottom of each tube in contact with the ice. Set a desiccator on a flat piece of dry ice in glycerol in a shallow metal container and pack the container with chopped dry ice. When the culture suspensions are thoroughly frozen and the bottom of the desiccator is cooled to −10°C. or less, place the tubes upright in the bottom of the desiccator. Make sure that each tube is in contact with the bottom of the desiccator. (See Fig. 13, p. 95.)

Place a large Petri plate of phosphoric anhydride on a support several centimeters above the tops of the tubes. Have all ground-glass surfaces of the desiccator thickly coated with a mixture of 1 part of paraffin and 6 parts of vaseline (*F80.2*), and close the desiccator. Keep the desiccator in the glycerol bath and place the whole apparatus in an insulated wooden box lined with galvanized metal as a protection in case of collapse and evacuate the desiccator to 500 microns or less of mercury. Store in the cold room for from eighteen to twenty-four hours. Remove to room temperature and check the pressure to make sure that no leaks have occurred. When the temperature inside the desiccator is equal to that of the room, reduce the vacuum slowly by opening the stopcock.

TABLE 3

Microorganisms stored in the dry state

Summary of methods used in preservation and distribution

CULTURE*	PRESERVATION		DISTRIBUTION: MEDIUM AND SPECIAL EXAMINATIONS†
	Dried suspensions prepared from 16- to 24-hr. cultures on‡	Comments	
Actinobacillus	Infusion agar (*F33*)		Methylene blue stain
Actinomyces—aerobic	Infusion agar (*F33*)		Acid-fast stain
Actinomyces—anaerobic	Infusion broth (*F3B*)	Incubate 1–3 days	Deep tube: infusion agar (*F35A*)
Bacillus	Infusion agar (*F33*)		
Bacterium—chromogenic	Infusion agar (*F33*)	Longer incubation at room temperature required for some species	
Bacterium—coliform	Infusion agar (*F33*)		Reaction in glucose-lactose agar (*F50B*); capsule stain, when required
Bacterium—enteric disease	Infusion agar (*F33*)		Reaction in glucose-lactose agar (*F50B*); macroscopic slide agglutination
Bacterium necrophorum	Chopped meat broth (*F6*)	Incubate 1–3 days	Deep tube: infusion agar (*F35A*) with 1-per-cent serum
Bacterium tularense	Cystine blood agar (*F35C*)		
Bacterium—miscellaneous	Blood agar (*F43A*)		Methylene blue stain, when required
Brucella	Infusion agar (*F33*)	Incubate in 5–10-per-cent CO_2	
Clostridium	Broth (*F6*, *F11*, or *F13.1C*)	Some species require incubation for 2 or more days	Deep tube: infusion agar (*F35A*, *F36B*, or *F45*)
Corynebacterium	Blood agar (*F43A*)		Methylene blue stain
Corynebacterium diphtheriae	Infusion broth (*F12*)		Coagulated serum (*F15*). Methylene blue stain
Hemophilus ducreyi	Infusion agar (*F33*) with 1.0 ml. rabbit blood on surface	Incubate 1–3 days; suspend growth in the blood on slant	

Hemophilus influenzae	Infusion agar (*F33*) with 0.2 ml. rabbit blood on surface	Suspend growth in milk (*F18A*)	
Hemophilus pertussis	Rabbit-blood agar (*F56B*)	Incubate 2 days; suspend growth in 5-per-cent rabbit blood broth (*F2A*)	Streak blood agar plate (*F56A*)
Lactobacillus	Milk (*F18A*)		
Mycobacterium	Blood agar (*F43A*)		Acid-fast stain
Mycobacterium tuberculosis	Egg medium (*F9*)	Incubate 1–2 months	“ “ “
Neisseria	Blood agar (*F43A*)		
Neisseria gonorrhoeae	Rabbit-blood agar (*F43A*)	Incubate in 5–10-per-cent CO_2; suspend growth in 5-per-cent rabbit blood broth (*F5A*)	
Neisseria meningitidis	Glucose serum agar (*F34C*)	See *N. gonorrhoeae*	Macroscopic slide agglutination
Pasteurella	Blood agar (*F43A*)		
Pneumococcus	Infusion broth (*F4*)	Incubate in 5–10-per-cent CO_2, when necessary; suspend growth in 5-per-cent rabbit- or horse-blood broth (*F4*)	Serum semisolid (*F36C*). Agglutination, solubility
Proteus	Infusion agar (*F33*)		Reaction in glucose-lactose agar (*F50B*); macroscopic slide agglutination (X-strains)
Sarcina	Infusion agar (*F33*)		
Staphylococcus	Infusion agar (*F33*)		
Streptococcus	Infusion broth (*F5A*)		Blood agar (*F43A*). Group precipitation
Vibrio	Infusion agar (*F33*)		

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* For method followed in the maintenance of strains in active use for the production of vaccines and sera—see respective sections in Methods of the Antitoxin, Serum, and Vaccine Laboratories.

† In general, dried cultures are distributed. When exceptions are necessary, the culture is grown on the medium listed under "Preservation" unless it is fluid.

‡ Suspensions are dried in a desiccator and sealed under atmospheric conditions except *Hemophilus ducreyi*, *H. influenzae*, and *Neisseria gonorrhoeae* which are dried by the lyophile process.

Remove the tubes from the desiccator and quickly examine the appearance of the dried material. If cultures resemble dry foam, immediately seal each tube by impregnating the upper portion of the cotton plug with cement (Pyseal) melted with a microflame. Reheat seals to remove air bubbles and to assure a tight union between the glass and cement; add more cement to provide a smooth layer over the cotton. If no evidence of contamination is obtained from the cultural and microscopic examinations of the concentrated suspensions, store the dried cultures at room temperature in drawers that are divided into small compartments, labeled, and indexed so that any strain is readily available. When subcultures are required, suspend the dried material in 0.5 ml. of broth (usually *F5A*) and use part of this suspension to inoculate suitable media immediately. Incu-

TABLE 4

Microorganisms stored in special media

Summary of methods used in maintenance and distribution

CULTURE	MAINTENANCE			DISTRIBUTION:* SPECIAL EXAMINATIONS
	Medium	Interval of transfer	Temperature of incubation and storage	
Fungi	Conservation agar (*F57.1A*) Honey agar (*F57.1C*)	3 mo.	room†	
Endamoeba	Egg albumen medium (*F16*) Asparagine serum agar (*F54*)	2 days	35°–37°C.	Moist preparation
Leishmania	Blood medium (*F54.5*)‡	2 weeks	room and 25°–27°C.	Moist preparation. Aerobic and anaerobic growth in broth (*F3B*)
Leptospira	Rabbit serum semisolid (*F34E*)	2 weeks	room and 25°–27°C.	Dark field. Aerobic and anaerobic growth in broth (*F3B*)
Treponema	Coagulated serum broth (*F2C*) Tissue semisolid (*F34D*)	6 mo.	35°–37°C.	See *Leptospira*
Trypanosoma	Glucose blood medium (*F43.5*)	2 weeks	room and 25°–27°C.	See *Leishmania*

* Cultures are distributed in the same medium used for maintenance.

† After growth at room temperature for 2–3 weeks, the cultures are stored at 4°–6°C.

‡ Medium *F43.5* may also be used.

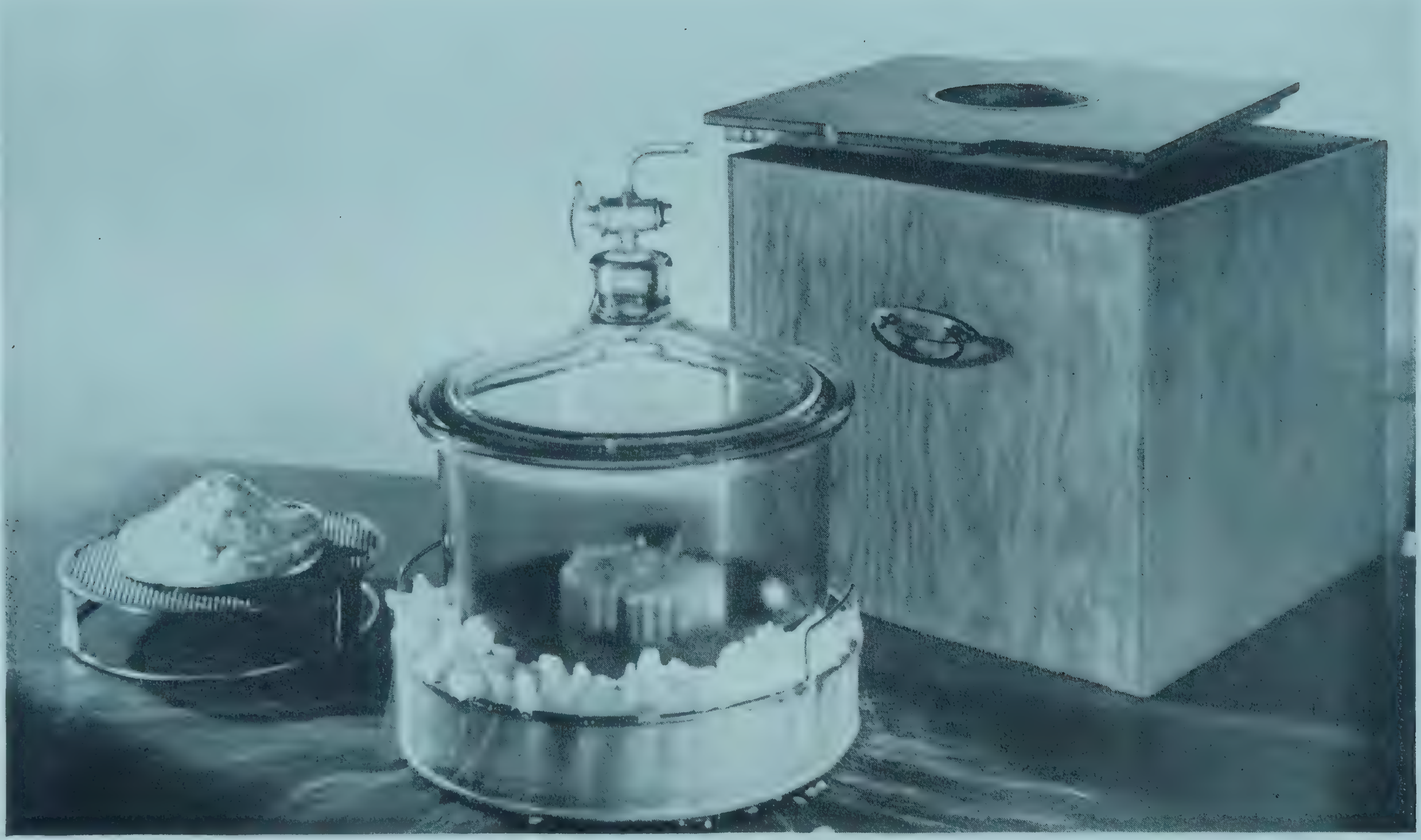

Fig. 13. Desiccator for Drying Cultures

bate the remainder and subculture again if the primary cultures fail to grow within from twenty-four to forty-eight hours.

The cement-sealed plugs may be loosened readily by the following procedure: Heat a fine metal rod with a small hooked and roughed point; push it through the seal, and rotate until the hook becomes enmeshed in the cotton plug. Then soften the cement by carefully heating the upper part of the tube and loosen from the glass by raising and rotating the metal rod.

Prepare additional dried cultures for storage as needed: subculture and redry new strains one year after receipt and other strains when only two tubes remain.

It is desirable to have on hand for each strain dried material prepared at different times, some of which has not been stored for more than two or three years.

If cultures are to be dried by the lyophile process, prepare concentrated suspensions and examine for purity according to the procedures already described. Dispense 0.05 ml. of the suspension in each of several 7- by 9.5-mm. tubes. To facilitate later sealing, constrict the tubes approximately 5 cm. from the bottom so that the inside diameters are reduced to about 2 mm. Prepare the lyophile apparatus for operation and connect the tubes to the manifold removing each cotton plug immediately before making the connection. Freeze the culture suspensions by immersing the tubes in a bath of dry ice and alcohol, and then proceed with the drying process which usually requires four or more hours. During drying check the pressure frequently. (If no appreciable leaks are present, pressures less than 250 microns of mercury are reached within fifteen minutes and are maintained throughout the period.) Seal each tube of dried material by fusion of the glass; during this operation check the pressure frequently to make sure that the vacuum is maintained. When all tubes are sealed, test the pressure in each by means of a high frequency spark coil. Select tubes in which the vacuum is satisfactory and the material has not foamed excessively due to thawing; store at room temperature or at 4°–6°C.

When subcultures are required, select a tube that has retained the required vacuum, cut off the fused end, and insert a sterile cotton plug. Make subcultures according to the procedure used for cultures dried in a desiccator (p. 94).

Storage in Beef-Infusion Gelatin and Special Media. The majority of cultures that are stored in beef-infusion gelatin (*F30*) or other media are maintained in duplicate; in the case of those stored at 35°–37°C., the two sets are kept in different incubators. The cultures are arranged in racks constructed to hold a double row of tubes. At one end a card is attached on which are recorded the number of the rack, the names of the species, and the number of each strain in the rack. In so far as practicable, related microorganisms that are similarly maintained are placed together in one rack.

When transplants for storage are to be made, select as seeds the unopened cultures of the last transfer, and from each inoculate two subcultures. Seal all tubes with paraffin or tie Cellophane over the plugs. Place the old transplants together in a metal basket marked with the rack number and store at least until the next transfer. After incubating the new cultures, observe carefully the macroscopic appearance of the growth. If contamination is suspected, examine microscopically and make additional tests for purity. Attach a second label to cultures that are opened and retained, and enter on it the date opened and the initials of the worker.

Use the following special procedures for cultures of fungi, *Endamoeba, Leishmania, Leptospira, Treponema,* and *Trypanosoma.*

Fungi: Select as seed a culture free from macroscopic evidence of contamination and pleomorphism (usually white tufts of mycelium). With a long stiff dissecting needle inoculate conservation agar (*F57.1A*) and honey agar (*F57.1C*) dispensed to provide a long slant and deep butt by placing the culture in small depressions made in the slant with the point of the needle. Grow the transplants at room temperature for 2–3 weeks. Seal the cotton plugs with paraffin; place the cultures in a tightly covered metal container; and store at 4°–6°C. For procedures to determine purity and identity see Chapter 31, p. 466.

Endamoeba: At least twenty-four hours before cultures of *End. histolytica* are to be transferred, add to each tube of medium (*F16* or *F54*) one small loopful of a mixture of one part of purified blood charcoal and two parts of rice starch sterilized by heating for one hour at 160°–175°C. in hot air. Incubate the tubes and, immediately before inoculation, examine macroscopically for contaminants. With a warm capillary pipette of wide bore, transfer some of the sediment from the base of the slant of the seed tube to the fluid covering the slant of the fresh medium. Return cultures to the incubator as soon as possible. Every week examine moist preparations from each strain for motile amebae. If available, use a microscope equipped with a warm stage for these examinations. (Occasionally the cultures are transferred daily or treated with neutral acriflavine, 50,000-fold final dilution to reduce the bacterial growth.)

Leptospira and Treponema: Examine moist preparations over a dark field and select as seed an actively motile culture. If contamination is suspected, examine preparations stained by Gram's method. In the case of *Leptospira*, remove with a pipette some of the growth from the seed culture and introduce it immediately below the surface of the medium (*F34E*). With strains of *Treponema*, take the inoculum from the upper area of growth in the seed culture and place it in the bottom of a tube of fresh medium (*F2C* or *F34D*) without introducing bubbles of air. Inoculate aerobic and anaerobic tubes of sterility-test broth (*F3B*) with the material remaining in the pipette after inoculation of the media.

Leishmania and Trypanosoma: Examine moist preparations and select as seed an actively motile culture. If contamination is suspected, examine preparations stained by Gram's method. If the tubes of medium to be inoculated have relatively little moisture at the base of the slant, add sufficient broth (*F5A*) to provide at least 1 ml.

of fluid. With a pipette, take up some of the fluid and sediment from the base of the slant in the seed culture and inoculate the tubes of fresh medium by allowing a small quantity of the fluid to run over the slant. For *Leishmania*, use medium *F54.5;* for *Trypanosoma*, medium *F43.5*. Inoculate aerobic and anaerobic tubes of sterility-test broth (*F3B*) with the material remaining in the pipette after inoculation of the media.

Passage through Animals.—Selected strains are maintained by passage through animals in order to retain pathogenic activities. The general procedures followed for the inoculation, marking, and post-mortem examination of the animals are described in the chapter on "The Use of Experimental and Test Animals," p. 73.

Mycobacterium tuberculosis var. *bovis* and var. *hominis:* Inject 0.5 ml. of suspension of culture or of minced tissue from an infected animal into the peritoneum of a guinea pig approximately 300 grams in weight. Examine the animal frequently and when evidence of infection develops, chloroform it, and remove the spleen to a sterile Petri plate. If evidence of infection does not appear within three months, chloroform the animal, and examine carefully for tuberculous lesions.

Remove a portion of the spleen containing tubercles; mince, and suspend it in salt solution (*F64*). Streak the suspension on blood agar (*F43A*) and examine stained preparations for acid-fast microorganisms. If they are present, inject another animal. Store the suspension at 4°–6°C. for at least one week so that if the culture taken at necropsy indicates the presence of contaminants, or if the animal dies of intercurrent infection, a second guinea pig may be injected. If the tissue is contaminated with microorganisms pathogenic for guinea pigs, treat the suspension with alkali as described for the isolation of the tubercle bacillus, p. 292, before injection of another animal.

Pneumococcus and Streptococcus: With sterile broth, so dilute the virulent culture, which has been grown for from twelve to sixteen hours in broth (*F4* or *F5A*) that from 10 to 100 minimum lethal doses are contained in 0.5 ml., and inject into the peritoneum of a mouse weighing 18–22 grams. When the animal dies, usually within forty-eight hours, perform a necropsy. With a loopful of the heart's blood inoculate media for storage—serum semisolid for pneumococci (*F36C*), beef-infusion broth for streptococci (*F5A*)—and before sterilizing the loop streak it on a blood-agar plate (*F43A*). Incubate the cultures for eighteen hours and store at 4°–8°C. until the next animal passage—usually one week.

Treponema pallidum: Wear rubber gloves and protect the eyes with glasses or goggles. On one operating tray fasten a rabbit with well-developed testicles; on another the infected rabbit. Dampen the hair in the inguinal region with 2-per-cent cresol compound (*F76*). Apply 95-per-cent alcohol to the scrota.

Draw 0.6 ml. of salt solution (*F64*) into a sterile 2-ml. syringe fitted with a $1\frac{1}{4}$-inch, 19-gauge needle. After the alcohol has evaporated from the scrotum, insert the needle into the center of the lesion of the infected rabbit. Inject rapidly part of the salt solution and immediately withdraw as much as possible. Inject approximately 0.2 ml. of this fluid into each testicle of the normal rabbit. From the remaining material in the syringe make a moist preparation and examine over a dark field.

In order to avoid loss of the strain, maintain two series of infected rabbits. Examine them at intervals of from one to two weeks and make the next animal passage when the lesions are well developed, usually from five to seven weeks after inoculation.

DISTRIBUTION OF CULTURES

All requests for cultures are referred to the collection group by or through whom all cultures are distributed. Cultures for the preparation of vaccines and sera are obtained from the department in which they are in active use and before distribution are examined in the collection group. Cultures for the preparation of autogenous vaccines and specimens for the control of technic that are sent to laboratories in the State are prepared by the Diagnostic Laboratories which are responsible for their purity. All cultures are shipped in accordance with postal or express regulations. The following highly pathogenic species are rarely distributed outside of the laboratory: *Bacillus anthracis*, *Bacterium mallei*, *Bacterium tularense*, *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Clostridium botulinum*, *Clostridium tetani*, *Pasteurella pestis*, and *Vibrio cholerae.*

When a request is received, ascertain that the laboratory is registered, see "Appendix B," p. 877. On the letter or order form requesting the culture, record the species and number of each culture, and the probable date of shipment. Enter on the distribution card the date, the name and address of the person to whom the cultures are to be sent, and the number of each culture; when the cultures are ready for packing, complete this record by entering the date of shipment.

Prepare and examine cultures in accordance with the general procedure outlined below and the detailed methods indicated in tables 3 and 4. When a dried culture is distributed, make sure that a duplicate preparation of the strain has been similarly examined. Include with the shipment directions for making subcultures from the dried preparation and a statement indicating that the culture was not examined immediately before packing.

I. From the stock culture remove at one time sufficient material to inoculate:
   1. Medium for shipment, usually the same medium as that used in growing cultures for storage.
   2. Broth or lactose-glucose agar (*F50B*) for differential tests.
   3. Plate of solid medium, usually (*F43A*).

   Inoculate two plates with anaerobic microorganisms for incubation under aerobic and anaerobic conditions.

II. Incubate the cultures (I) under optimum conditions for growth.
III. Make a film from the culture to be distributed (I, 1), stain by Gram's method, and examine thoroughly.
IV. Note the results of special tests (I, 2).
V. Examine carefully the colonies in the plate (I, 3).

VI. If the various examinations indicate that the culture (I, 1) is pure and has the properties characteristic of the species, seal the plug with paraffin.

When a culture in semisolid medium is to be shipped, add sufficient agar (*F35A*) that has been melted and cooled to 45°C. to form a layer approximately 1 cm. thick over the culture medium. If a culture in fluid medium is to be distributed, or if a culture in solid or semisolid medium is to be in transit for a long time, seal the tube in a flame.

VII. To each tube attach a gummed label on which the name of the laboratory is printed. Enter the following information on the label and check with the data in the accession book and on the distribution card.

1. The name of the species, the number of the strain, and the date of preparation.
2. The name and address of the person to whom the culture is sent.

If the culture is to be used for the preparation of autogenous vaccine, a second label is attached on which the names of the patient and of the attending physician are recorded.

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CHAPTER 7

DETERMINATION OF HYDROGEN-ION CONCENTRATION

Hydrogen-ion concentration is usually expressed in terms of pH. This was originally defined as the logarithm to the base 10 of the reciprocal of the hydrogen-ion concentration in normality.

$$\mathrm{pH} = \log \frac{1}{[\mathrm{H}^+]}$$

For a detailed discussion of the theories underlying this subject the reader is referred to Clark (1) or other authorities.

The electrometric method is used in this laboratory for the standardization of buffer solution and for special problems; the colorimetric for routine work.

THE ELECTROMETRIC METHOD

Electrometric determinations are made with a reliable, commercially available glass-electrode assembly. The electrode consists of a thin-walled bulb made of special glass, on the inside of which a suitable electrolyte and an auxiliary electrode are placed. The calomel reference electrode is of the saturated type. Both electrodes are immersed in the solution to be tested and connections are made with the potentiometer circuit. The instrument is calibrated in pH units and readings are made directly. When not in use the glass electrode should be kept immersed in distilled water and not allowed to dry.

THE COLORIMETRIC METHOD

The colorimetric method is based on the fact that stable buffer solutions can be prepared which cover the range of pH values generally used, and that when the proper indicators are added to these solutions, a graduated series of colors is obtained. These colors serve as standards with which to compare unknown solutions treated similarly. A buffer solution is one that exhibits a resistance to change of pH when it is subjected to gain or loss of acid or alkali. The buffer value (β) of a medium at a specified pH is defined by Van Slyke (2) as the gram equivalents of strong alkali (e.g., moles of NaOH) required per liter of medium per unit change in pH. For example, in the titration of a medium, a rough estimate of the buffer value is usually possible from the data so obtained. Let Δ pH be the change in pH brought about by addition of t ml. of N NaOH to 100 ml. of medium ($10 \cdot t$ ml. per liter). Then the buffer value,

$$\beta = \frac{10 \cdot t}{1000 \cdot (\Delta \text{pH})} = \frac{0.01 \cdot t}{\Delta \text{pH}} \text{ (approximately)}$$

Where acid is required, the titer (t) has a negative value. However, as Δ pH is then negative, β is always positive. The buffer mixtures and indicators are prepared as recommended by Clark (1). Buffer solutions should be stored in flasks of pyrex glass, tightly stoppered to prevent contamination from the carbon dioxide of the air.

Procedure.—The essential points of the colorimetric procedure are the addition of a suitable indicator to a series of tubes containing buffer solutions and to a similar tube containing the unknown. The pH value is then determined by matching the colors. The relative volumes of indicator and test solution may be varied to meet conditions but must be the same in matched tubes, standard and unknown. In general, 0.4 ml. of indicator and 10 ml. of solution give a satisfactory depth of color. In the titration of culture media, however, it is more convenient to use 0.5 ml. of indicator and 15 ml. of solution. Thus a medium that is darkly colored before indicator is added, or one that is solid or viscous (e.g., agar), is conveniently diluted threefold, as described in "Preparation of Media," p. 173. See Clark, p. 40 (1). With colored or turbid solutions, such as culture medium or serum, a comparator made from a block of wood is used. Six deep holes, large enough to hold tubes of a suitable size, are bored in two parallel rows of three holes each. Adjacent pairs are placed with intervals of 2-3 mm. between holes. At right angles to these holes and running

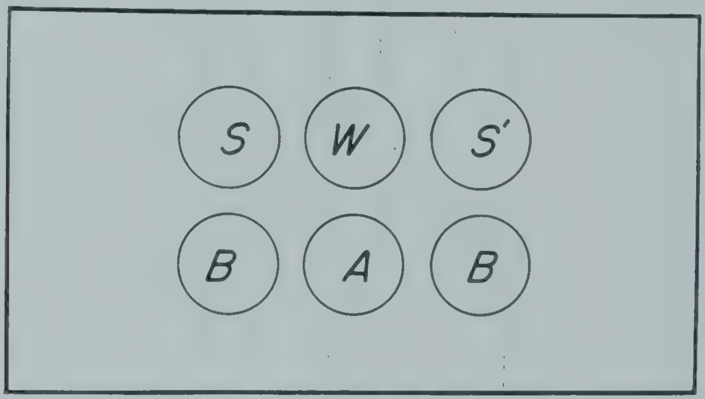

FIG. 14. POSITION OF TUBES IN COMPARATOR BLOCK

W = water
S, S' = standard buffers with indicator
A = unknown solution with indicator
B = unknown solution

through each pair are bored smaller holes or preferably rectangular slots through which the tubes may be viewed. The holes or slots are covered by a frosted-glass window which may be conveniently fastened to the back of the block. The block, including the insides of the holes, is stained black. The position for the tubes in the comparator is shown in Figure 14.

In making comparison a suitable artificial light-source is of great aid. Inexpensive and fairly satisfactory is a 60-watt, daylight, electric bulb placed in a dark vented box about 25 cm. from the objective aperture.

Errors of the Colorimetric Method

Aside from color and turbidity, other difficulties of the colorimetric method are the so-called protein and salt errors. These may sometimes be obviated by dilution but in any event it is wise to estimate the error by a comparison of the colorimetric and electrometric determinations. If necessary, proper allowance should be made for the error.

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CHAPTER 8

GASOMETRIC CHEMICAL ANALYSIS AND RELATED TECHNICS

The methods of gasometric analysis furnish an approach to the general subject of quantitative chemical analysis on the same level as the classical methods of gravimetric and volumetric analysis. The present purpose is, first, to indicate their general nature and scope and, second, to bring together certain modifications and auxiliary methods that have been found useful. The wet combustion method for carbon determination, together with its applications to elements or radicals after precipitation in organic combination is discussed first because it serves both purposes. The method for study of primary amines by the nitrous acid reaction using a special apparatus, and the newer manometric technic for the same purpose have found wide application. Important recent modifications are indicated, as is also the newer method for free α-amino acids based on the more specific ninhydrin carbon dioxide reaction, for which the term "carboxyl nitrogen determination," is preferred (1, 2) to avoid confusion with the amino nitrogen determination by the nitrous acid reaction. The gasometric determination of hemoglobin as a basis of standardizing other methods is discussed. Methods developed for estimation of sugars in various combinations and in media by direct live-yeast fermentation and measurement of the carbon dioxide are also indicated.

Auxiliary technics, such as quantitative ultrafiltration, use of a glass rack for drainage of precipitates in centrifuge tubes, and use of safety pipette fillers have much wider potential applicability than that indicated.

In so far as possible, references in the literature to the actual procedures have been used rather than to give details of technic.

In gasometric analysis the portable form (3, 4) of the Van Slyke-Neill (5) apparatus with the Van Slyke-Folch chamber (6) is used for vacuum extraction and manometric measurement to determine various substances which are gases or may be made to react directly or in a sequence of reactions resulting in formation of a gas suitable for measurement and quantitatively related to the original substance in question. The function of the manometric apparatus in such cases is analogous to that of the macro- and microanalytical balance in gravimetric determinations.

Manometric Carbon.—An excellent example is the manometric carbon determination (6) by combustion of organic compounds with the aid of a mixture of chromic, iodic, sulfuric, and phosphoric acids, with transfer of the CO_2 to the chamber and its simultaneous absorption there in an alkaline hydrazine solution, followed by its liberation and measurement. The carbon may be determined by this method of *wet combustion* with an average deviation about 0.2 per cent in macroanalyses (with 8 to 15 mg.) or in microanalyses (with 2 to 3.5 mg.) and about 0.5 per cent in submicroanalyses (with 0.3 to 0.7 mg.). Yields of carbon from most organic substances are within a few parts per thousand of the theoretical values (6). The technic has found extensive use in this laboratory, among other things, for studies of the composition of cardiolipin and products of its hydroylsis. For auxiliary microgravimetric determination of solids in solutions see chapter on "Other Methods of Quantitative Chemical Analysis and Precision Tests," p. 117.

A centrifuge-combustion tube has been developed, the best form being that of Van Slyke and Kreyse (7), in which all the operations of precipitation, centrifugation, decantation, and drainage, successive washings of the precipitate with centrifugation and drainage each time, drying, and combustion may be performed. The tube is designed for connection by a ground joint and a delivery tube to the manometric apparatus, as usual (6), and is suitable for direct heating with a microburner having a chimney a little over three inches long.

Rack for Decantation Drainage.—An all-glass rack, made from Pyrex rod (8 mm. diameter), bent and fused in a form somewhat resembling a music stand, has been devised to hold the tubes during drainage without contact with other materials (8). This is especially valuable where carbon determination is the ultimate means of measurement, but may be useful in any case where precipitation and washing are performed in centrifuge tubes.

Three rods 300 mm. long are held parallel in a frame (formed by suitable bending and fusing), the axis of that at the top being 100 mm. from that of the middle rod which is 22 mm. from the axis of the third. Two planes, both passing through the axis of the middle rod and respectively through the other rod axes, would form an angle about 100° to 110°, the rods thus forming a ledge and back. In the middle of the top rod is fused another rod at right angles and directed so as to form a leg for the rack. The end of this is hooked slightly and formed so that it may rest on an Erlenmeyer flask (weighted with shot, and about 85 mm. high—50-ml. capacity), so that the back of the rack is about 70° from horizontal (the ledge being then about horizontal); but the leg used alone as a prop will hold the back of the rack at 30° from horizontal.

In some technics (7) drainage of the tubes for a short time at 30° and then nearly vertical is required. This can be done without disturbing them on the rack as is illustrated in figures 15A and 15B. To keep the tops of the tubes from sliding and toppling, as may occur if the rack is slightly jarred, it is convenient to cut little open rubber rings from nitrometer tubing, which are slipped over the top rod of the rack as spacers for the tubes. Glass lugs may be fused on the rod for this purpose, or the rod may be beaded in the first place by heating and compressing it at convenient intervals before forming the rack.

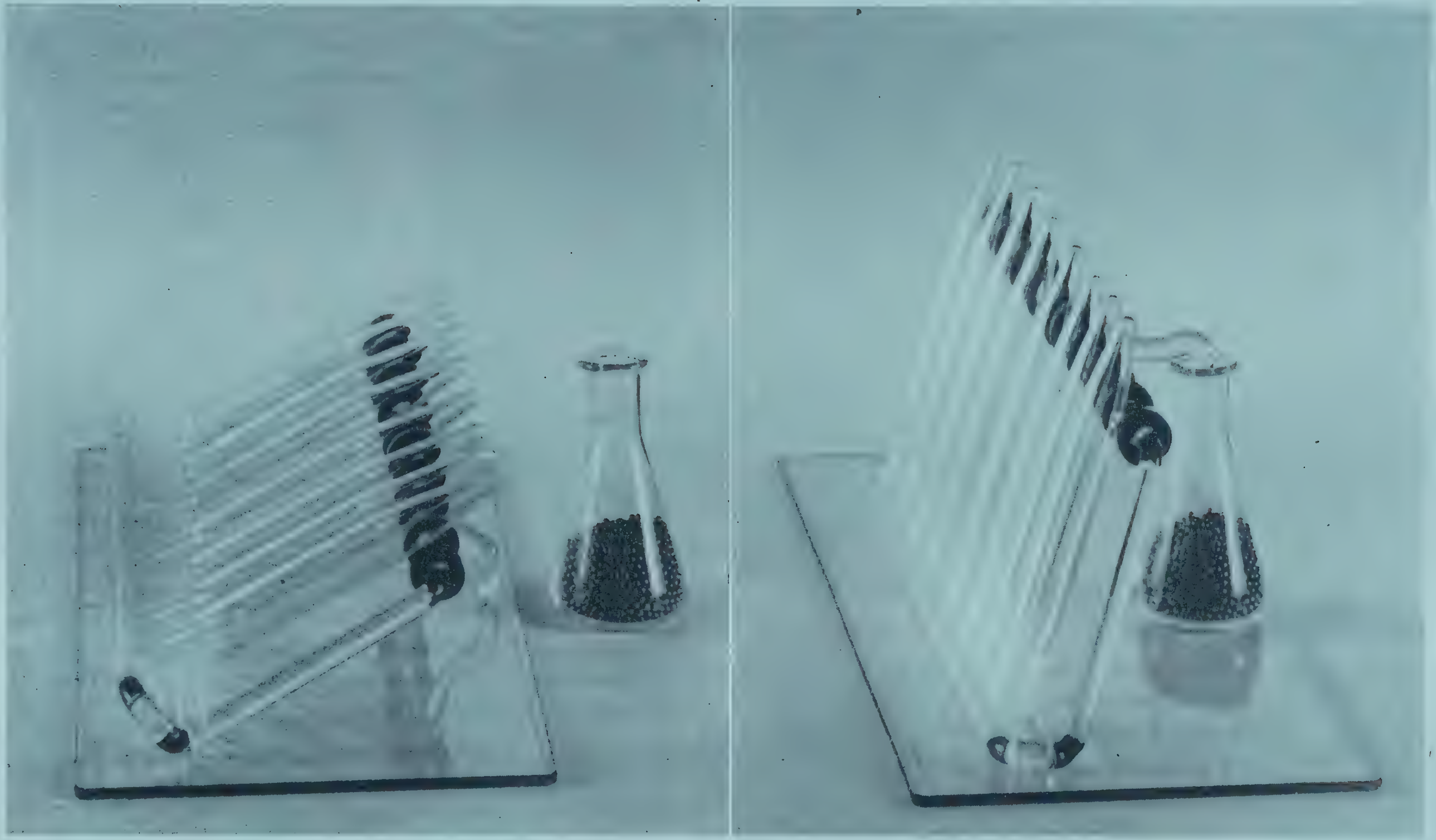
Elements or Inorganic Radicals as Organic Precipitates.—The technic of precipitation in an organic compound and determination by wet combustion has been used as the basis of many methods of determination of elements or inorganic ions, following Kirk's method (9) for phosphorus in inorganic or organic original combination which was adapted by Hoagland (10) to essentially the technic outlined above except for the use of the more satisfactory form of centrifuge-combustion tube later developed (7). Procedures of this type for determination of sulfate (10), magnesium (11), and calcium (7), as well as phosphorus, are now available as provisional methods for special studies. Some use has been made of them (12) and also of the general plan in studies of the conditions of precipitation and washing of organic precipitates (8).

Safety Pipette Filler (13). (Special Uses).—For use with combustion fluid, as in the wet-combustion technic (6), a glass bell dust shield is attached at T around the rubber connection of the safety pipette filler, described previously, see "General Bacteriologic Technic," p. 8. The pipette may be left attached to the filler and inserted in a long glass tube with closed bottom (test-tube form) of suitable size to permit the glass bell to rest upon and cover the top, suspending the pipette above the bottom or any collected fluid. This provides protection from dust in intervals between use.

It is convenient also to use a filler with stopcock pipettes (4), and to leave one attached to any pipette used frequently by different workers.

"Carboxyl Nitrogen" (Free α-Amino Acids).—The ninhydrin reaction is used to liberate CO_2 from carboxyl groups of free α-amino acids, and the CO_2 is transferred to the Van Slyke chamber and there determined. The ninhydrin-carbon dioxide method of Van Slyke and others (1) or the modification of Hamilton and Van Slyke (14) for blood filtrates after protein removal with picric acid, is used for estimation of "carboxyl nitrogen" as defined in the earlier paper (1) and now preferred by the authors (2). The all-glass reaction vessel (14) has been adopted in all cases, as well as the lactic acid and NaOH solutions nearly saturated with NaCl. The appropriate general factors for use of these NaCl-containing solutions are given by MacFadyen (15); those given by Hamilton and Van Slyke take into account the several special conditions of precipitation of proteins and consequent dilution of the original material.

Primary Amino Nitrogen (Nitrous Acid Reaction).—The reaction of ali-



A

B

Fig. 15. Rack for Holding Glassware for Draining Precipitates in Combustion-Centrifuge Tubes. A. Rack Resting on Its Leg. B. Rack in Upright Position

phatic amino groups with nitrous acid according to the equation $R \cdot NH_2 + HNO_2 = ROH + H_2O + N_2$ is the basis of a method of Van Slyke which originally employed a special apparatus for the purpose (16), later improved (17) and developed for microanalyses (18–20). A simplified and improved technic (20) was given for preliminary removal of air from the apparatus. The 30-per-cent sodium nitrite solution should contain 0.5 per cent of KI as recommended by Kendrick and Hanke (21). Koch's (22) modification of the apparatus may be employed. The modified micromethod is used for routine analyses.

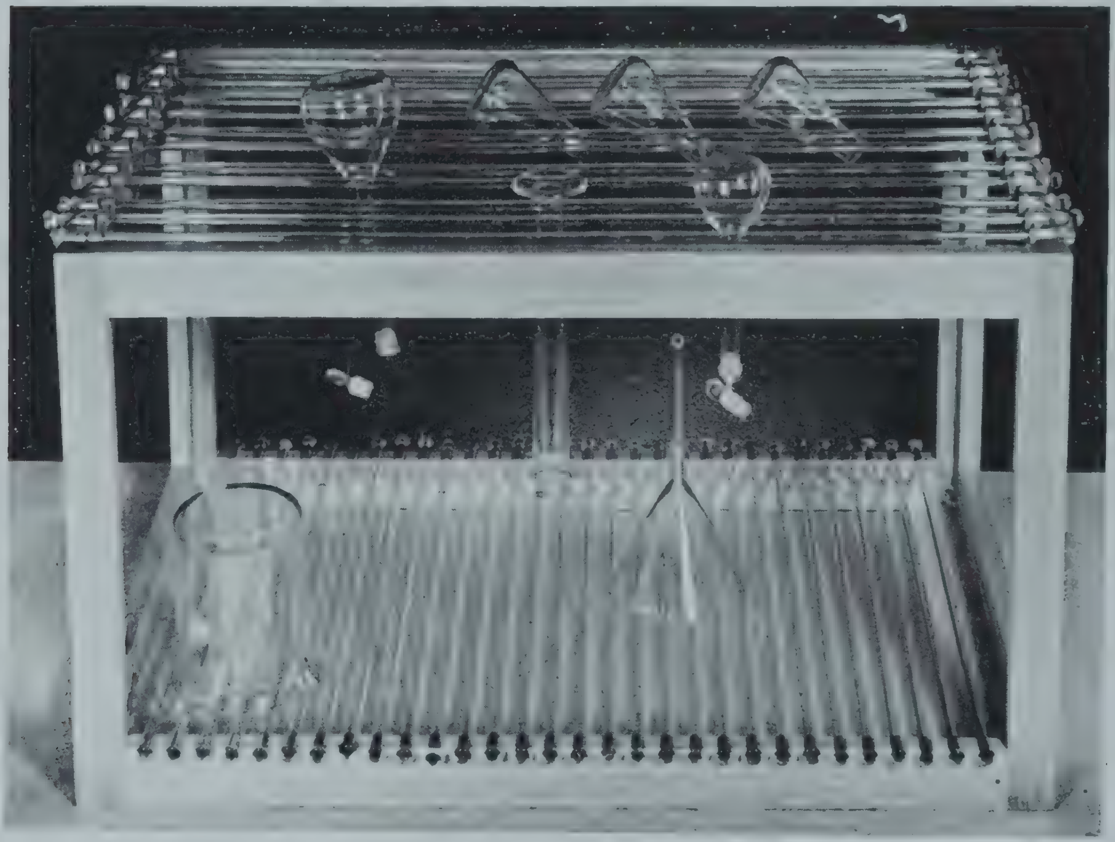


FIG. 16. GLASS RACK FOR DRYING APPARATUS

A method using the Van Slyke-Neill (5) manometric apparatus was developed subsequently (23) and a modification (21, 24, 25) of this technic is available for the same purpose. Moreover, the manometric method has a sensitivity about 0.0005 mg. of amino nitrogen and permits analyses of samples as large as 5 ml.; whereas that employing the older special microapparatus has a sensitivity about 0.002 mg. N and may use at most a 2-ml. sample. This permits direct use of solutions in the manometric technic that otherwise would require a 10-fold preliminary concentration, and four analyses may be made on the material otherwise required for one.

The modification of the manometric technic has been adopted in Dr. Van

Slyke's laboratory (24) since publication of the original article (23). The essential difference (21) is to include 1 gram of potassium iodide per 100 ml. of the sodium nitrite solution (800 grams $NaNO_2$ dissolved in 1 liter of water) and use of 5 per cent of sodium thiosulfate in the first wash water after the analysis, as suggested by Kendrick and Hanke (21, 25). In the improved technic (24) fresh alkaline permanganate is used in the modified Hempel pipette each day, equilibrated with gases from a dummy run before use in analyses.

Hemoglobin.—The oxygen-capacity method of Sendroy (26), modified by Sendroy, Dillon, and Van Slyke (27), is used in standardization of other hemoglobin methods, but it is preferable (24) to use 1 ml. of air-free 0.5 *N* NaOH solution for absorption of CO_2 (instead of *N* NaOH) because the 0.5 *N* alkali floats on the top of the liquid, protecting the O_2 from reabsorption by the blood remnants and also gives a clearer meniscus. It should be noted that blank analyses are made on 3.5 ml. of 0.9-per-cent salt solution (rather than 3.35 ml.) and corrected tables are available (27, 4a) for estimation of physically dissolved O_2. An alternative gasometric method is that of Van Slyke and Hiller (28) which is preferable where the reagents and space for their maintenance are available. A pure supply of carbon monoxide is used instead of oxygen in air in determinations of combining capacity. The O_2 and CO reactions with hemoglobin are similar but the latter is preferable, if blood samples are used for gasometric analysis in comparison with analyses of the same specimens by other methods as a basis of their standardization. If the oxygen capacity method is used, the blood specimens should not be drawn from smokers, who may have as much as 1 volume per cent of CO in their blood (4b); this would be equivalent to roughly 5 per cent of the normal capacity of adults and would lead to a corresponding error in hemoglobin estimation by the oxygen-capacity method.

Sugars in Various Combinations in Biologic Media.—A method has been developed (29, 30) which is based on the same central idea as Holden's method (31) for estimation of blood sugar by simultaneous fermentation with live yeast in the vigorously shaken Van Slyke-Neill (5) chamber, and extraction of the carbon dioxide formed, which is then determined manometrically. In our experience with Holden's method the presence of air in contact with yeast before and during fermentations was found (29) to be a serious source of discrepancies, both in blank analyses and in fermenting power of the yeast suspensions. The principal modification is the use of nitrogen gas to eliminate air in the yeast suspension and all reagents (including the sample material) which enter the fermentation mixture. The distilled water used to wash the apparatus is also washed with nitrogen. A revised timing scheme permits longer periods for fermentation, yet by use of three manometric machines by two workers (or two by one worker) determinations can be made at the rate of four or five per man-hour. The method is more suitable where many analyses are to be made than where they are only occasionally required, and for special investigations; such as, determination of glucose in maltose preparations or complex media, fructose in the presence of inulin, or the extent of partial hydrolysis of lactose (29, 32), inulin, or maltose. As little as 0.1-per-cent hydrolysis is measurable. A simple preliminary hydrolysis technic permits estimation of the polyose after its completion (29).

Quantitative Ultrafiltration in Chemical Analysis.—In the case of complex media that produce excessive foaming during fermentation, a method of quantitative ultrafiltration (30) is available. This permits removal of foam-producing substances without excessive dilution (otherwise usually 10-fold with protein defecants). Indeed, simultaneous concentration may be effected. The technic should find many applications in other types of chemical analysis.

The ultrafilter consists of a nitrocellulose membrane formed inside a coarse alundum crucible, used in a funnel as in ordinary vacuum filtration; or the membrane may be used instead in a funnel provided with a sintered filter. For methods of preparing the membranes see "Ultrafiltration," p. 823. The funnel leads directly to a calibrated tube through a rubber stopper provided also with a delivery tube for attachment to a vacuum line. If evaporation of the filtrate is to be promoted during the filtration, a capillary tube inlet, controlled by a stopcock or rubber tube with a Hoffman clamp, may be adjusted so as to play a fine jet of air on the surface, and the collecting tube may be immersed in a suitable bath if temperature elevation is permissible; however, these operations are not usually required.

The filter is given a preliminary washing and the washings rejected. Several equal volumes (V) of sample material are measured in succession in the same ultrafilter, but each only after the contents of the crucible have been diminished to about one-fifth of V. Then two like volumes of water are introduced in succession in the same manner. After the remnant in the filter has been reduced finally to $V/5$ or less, filtration is stopped, the vacuum released carefully, the filter removed, and the filtrate made up to volume with adjustments as required (for example, addition of indicator and citric acid for the fermentation method). Berkefeld filtrates of samples of media in which cultures are growing may be refiltered thus through nitrocellulose membranes preliminary to determination of glucose. This is made the basis of replenishments of glucose in the medium during the course of growth.

Glass Rack for Drying Apparatus.—A rack made of a wooden frame, paraffin finished, with horizontal glass rods is used to permit drainage and drying of glassware without contact with other material that might affect sensitive chemical analyses such as the manometric carbon determination (Fig. 16). Such racks are used extensively throughout the laboratory. An upper layer of rods is held securely by stout rubber bands, such as old preserve-jar rings. The rods lie in semicylindrical grooves, 7/8 inch apart in the lower, 5/8 inch apart in the upper level; the rods may be spaced at variable intervals according to usage.

For drying tubes a greatly added convenience is provided by use of other rods parallel to those at the lower level but several inches higher. These are supported by two parallel legs at the ends made by bending the rod at right angles and by a third leg joined perpendicularly at the middle but making an angle about 90° with the other legs. Such a rod and those of the lower level form a rack for tubes similar to the one-piece rack used for combustion-centrifuge tube drainage during analyses, Figs. 15A and 15B.

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CHAPTER 9

OTHER METHODS OF QUANTITATIVE CHEMICAL ANALYSIS PRECISION TESTS

Most of the chemical analyses made as a basis of control of production or standardization of diagnostic, prophylactic, therapeutic, and other biologic products are based on methods readily available in the literature.

A summary of methods in frequent use is given in this chapter, together with more detailed descriptions of certain modifications and additional methods that have proved useful. A brief discussion of simple methods of testing analytical precision is also included.

Unless otherwise noted, microgravimetric technic is in accord with that given in the text of Niederl and Niederl (1), based on the earlier work of Pregl, and other technics as in that of Peters and Van Slyke (2). Standard acid and alkali solutions are based on comparison with Hulett and Bonner's constant boiling hydrochloric acid (2-a).

ESTIMATION OF PRECISION

Obviously, theories of error cannot be expected to correct errors due to mistakes. These should be controlled first by development of habits of care by the technician, and, second, by independent checking by an experienced worker as much as practicable. Certain types of tendencies to error may be compensated or a correction may be applied in calculation. Residual errors beyond such control are considered in theories of error and their probable influence estimated.

Errors in Instruments and Standards.—Carefuly calibrated apparatus is usually designed to give measurements with errors within prescribed tolerances that are small enough to be neglected in ordinary usage. However, for precise work a calibration correction may be added algebraically to the nominal value of the instrument or a calibration factor may be used in some cases. Standard solutions usually have such a factor (f) slightly different from unity. Possible effects of accumulation of errors in volumetric analysis are discussed by Peters and Van Slyke (2-b).

The precision of instruments and technics in all analytical work should be checked by a series of observations (X_i) under prescribed conditions, where $i = 1, \cdots, N$. A precision study of microbalances in various laboratories has been reported (3) by a committee of the American Chemical Society, and a recent article (4) written for chemists discusses some relatively simple statistical methods that should be applied to estimation of precision of chemical analyses.

Calculation of Variance and Standard Deviation.—For such a set (X_i) of N observations, whose mean is $\bar{X} = \Sigma X/N$, and deviations $d_i = X_i - \bar{X}$, the standard deviation estimate (s) for individual observations is given by $s^2 = \Sigma d^2/(N - 1)$. s^2 is the *variance* and the formula is that now usually employed (5, 6), or the equivalent formula $(N - 1)s^2 = \Sigma(X - C)^2 - N(\bar{X} - C)^2$, where C is an arbitrary constant. The standard deviation of the mean is estimated by $s_{\bar{X}} = s/\sqrt{N}$.

Example. Required to calculate the standard deviation of a set of three dry weight values obtained in estimation of solute in a given volume of a solution; respectively 6983, 6990, and 6992 micrograms (each estimate being the mean of several weighings). Then we have $X_1 = 6983$, $X_2 = 6990$, $X_3 = 6992$, and $N = 3$; whence

$$\bar{X} = \frac{6983 + 6990 + 6992}{3} \cong 6988 + \tfrac{1}{3} \cong 6988.33.$$

Then approximately

$$\begin{aligned} d_1 &= 6983 - 6988.33 = -5.33, & d_1^2 &= 28.4089,\\ d_2 &= 6990 - 6988.33 = 1.67, & d_2^2 &= 2.7889,\\ d_3 &= 6992 - 6988.33 = 3.67, & d_3^2 &= 13.4689. \end{aligned}$$

$$\text{Approximately } \Sigma d^2 = 44.6667,$$

$$s^2 = \Sigma d^2/(N - 1) \cong 44.6667/2 \cong 22.3333.$$

$$s \cong \sqrt{22.3333} \cong 4.73.$$

However, the calculation is easier taking C conveniently for example as 6988. Then the values of $(X_i - C)$ are −5, 2, and 4, obviously; and their squares are 25, 4, and 16, respectively. The sum $\Sigma(X - C)^2 = 45$, and $N(X - C)^2 = 3(6988 + \tfrac{1}{3} - 6988)^2 = 3(\tfrac{1}{3})^2 = \tfrac{1}{3}$.

$$\therefore s^2 = (45 - \tfrac{1}{3})/2 = 22 + \tfrac{1}{3}$$

$$\text{and } s = \sqrt{22 + \tfrac{1}{3}} \cong 4.73 \text{ micrograms,}$$

$$\text{and } s_{\bar{X}} = s/\sqrt{N} \cong 2.73 \text{ micrograms.}$$

The reliance to be placed on standard deviation estimates depends, of course, on the number of observations (5, 6).

Cumulative Frequency Method.—A simple alternative method of studying variation has been developed (7, 8) which has the further advantage of avoiding any prior assumption as to the character of the variation (distribution form) except that no two values exactly alike are supposed to occur. In actual practice by processes of *rounding off* values as in reading an instrument, values just alike are often recorded. In that case the treatment may be regarded as only approximate to that extent. A set of N

random observations (X_i) as above is simply arranged in ascending order of magnitude $X_1 \leq X_2 \leq \cdots \leq X_N$.

Assuming this and that the future observations that can be made in the same manner are unlimited in number, it has been demonstrated that, if any such future observation (X) is made, then the probability $(\bar{p}_k)$ that X is less than X_k is exactly $k/(N+1)$ as judged by the available evidence. $\bar{p}_k = k/(N+1)$. The successive values of X_k may be plotted against the corresponding values of $\bar{p}_k$ on coordinate paper to form a graph. This system has been employed in a study (9) dealing incidentally with relative values obtained in estimation of phosphorus in the same materials by two different chemical methods.

Rejection of Observations.—The rejection of one or more outlying observations from a set often appears desirable. Wherever it is known that there was an opportunity for unusual sources of error to influence certain observations, they may be discarded provided that a decision to discard such observations is made without knowledge of the value obtained, preferably prior to completion of the observation and irrevocably regardless of whether the value later appears to be *satisfactory*. When rejections are to be made otherwise a strictly objective criterion is required which takes into account the extent of information available. The tau (τ) criterion (10) was designed to meet these requirements in dealing with such a situation occurring in an environment of precise laboratory work but where the standard deviation must be estimated from variations within a *small sample* of replicate observations containing the observation or observations in question.

An article by Pearson and Sekar (11) is devoted to a discussion of the τ-criterion, and criteria previously proposed have been reviewed by Rider (12). Independently, Arley (13) also obtained the same (tau) distribution as was used for the rejection criterion.[1]

Trends.—Often results obtained in simultaneous observations are found to be far less variable than corresponding observations not made simultaneously. One such situation occurs in microgravimetric determination of the mass of solids within closed vessels such as Parr weighing bottles. This may be due largely to variation in buoyancy effects—the Parr bottle being filled with dry air whereas the balance-case air, which it displaces, is humid and consequently less dense. Thus at 25°C. the mass of 10 ml. of dry air would be about 137 micrograms greater than that of an equal volume of air saturated with water vapor (100-per-cent relative humidity). The difference in mass for any other relative humidity, denoted by *R* in per cent, would be approximately 1.37 *R* micrograms. At 20°C. it would be about 1.03 *R* micrograms. Thus a change from 50- to 75-per-cent relative humidity between two weighings would result in an apparent increase in mass in the Parr bottle amounting to about 21 to 34 micrograms for temperatures between 20° and 25°C. To avoid such errors a compensating procedure may be used (see p. 117).

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[1] His table 1 is essentially the same as Thompson's (10) table 2 of $\tau_{(P,N,1)}$, but is extended to give values for $P = 0.001$ and certain values of f (equivalent to Thompson's $N - 2$) = 35, 40, 45, 50, 60, 70, 80, 90, 100, and 120. The two tables are in substantial agreement. A comparison indicates that the only important differences are that in Arley's table 1 the value for $f = 13$ and $P = 0.6$ should be 0.552 instead of 0.550 and that in Thompson's table 2 the value for $N = 4$ and $P = 0.05$ should be $1.645\underline{5}$ instead of 1.6080.

MICROGRAVIMETRIC TECHNIC

Minimizing Sources of Error in Microweighing.—In use of a microbalance great care should be exercised to avoid sudden variations in temperature and humidity; for example, by never exhaling in the direction of the balance case—the best practice seems to be to turn the head to one side and exhale through the mouth quickly. Zero readings on the balance should be taken preferably immediately before and after each weighing or at least at the beginning and end of a short series of weighings. The zero value used should then be estimated by simple interpolation.

Approximate Elimination of Certain Buoyancy Effects.—It is a common practice to use counterpoises, often kept in the balance case, for each weighing vessel used in current microgravimetric work (1, 14). The counterpoise is usually adjusted to about the same mass as the corresponding weighing vessel by use of lead shot. It is a better practice to use material of the same density throughout or about the same average density so that the air displacement volume of the counterpoise is also nearly equal to that of the weighing vessel, exclusive of gas spaces within the vessels. To the same end it is important to avoid completely enclosed air spaces in bottle handles, as by use of flat-topped covers without handles, because their air displacement is difficult to compensate. These buoyancy differences will affect the results, where displacement volumes differ, since air density varies from time to time with barometric pressure and humidity. The humidity effects, previously discussed (cf. also p. 117) are independent of barometric pressure, depending only on the humidity of balance-case air and the capacity of the enclosed air space of the weighing bottle.

METHODS

Moisture.—Usually moisture is estimated gravimetrically by heating 10- to 15-mg. samples in a platinum boat in an oven at 105°–107°C. for two hours or to constant weight.

Solid or Other Residual Solute Weight.—If the material to be determined is relatively stable, it may be heated in platinum boats or other suitable containers in an oven at 105°–107°C. for 15–18 hours or more to constant weight. Material that is readily oxidized in air may be treated in an Abderhalden dryer in an atmosphere of nitrogen at an appropriate temperature and pressure with the aid of a suitable desiccant, such as phosphorus pentoxide or possibly barium oxide (15). The temperature of drying may be varied by the choice of different solvents.

NOTE: The material for chemical determinations is frequently best obtained in solution, but the analytical result is required in terms of weight of the solute. In such cases the technic above or that following may be used as indicated by the circumstances.

Control Compensation in Microgravimetric Determination of Solids (16).—Samples are pipetted precisely into Parr bottles, previously dried and tared. Corresponding to these bottles, other bottles of approximately the same size without sample are used as controls. Variations in apparent mass occur in all bottles as a result of their being filled with dry air and bathed in moist air in the balance case during weighings, the humidity of the balance-case air being different on different occasions with corresponding differences in buoyancy effects (See *Trends*, p. 115). The variation in apparent mass of the controls is applied as a correction by subtraction from the apparent sample weights.

Obviously, it is not necessary to have the controls approximate the mass of the sample bottles closely as is the case with counterpoises. They are readily cleaned in the same manner as the sample vessels, and treated likewise throughout. Furthermore, there need not be a one to one correspondence between controls and sample bottles; a few controls may serve adequately for many sample bottles treated simultaneously.

Ash.—Solid samples between 0.02 and 2.0 grams are heated in platinum, quartz, or porcelain boats or dishes in a micromuffle tube (1) or muffle furnace at 600°C. for a half hour. If necessary, the cooled sample material may be treated with one or two drops of water, dried carefully, and reheated at 600°C. Treatment should be sufficient to bring the residue to constant weight.

Liquid samples, 1.0 to 2.0 ml., are precisely pipetted into platinum or porcelain crucibles. They are heated carefully to evaporate the solvent, and then put in a muffle furnace at 600°C. for a half hour.

Formaldehyde, Gravimetric Method.—Formaldehyde is determined by the method of Velluz (17) as modified by Wadsworth and Pangborn (18).

Phosphorus in Organic Compounds, Gravimetric Method.—

A. Dry samples are weighed in silver crucibles of about 15- to 20-ml. capacity preferably in amounts containing about 0.3 to 0.7 mg. of phosphorus. To this is added 1 ± 0.1 gram of KOH and about 0.2 gram of KNO_3, and the material is fused according to the method of Elek (19) except that the crucible is covered with a silver lid to prevent loss by spattering.

B. Liquid samples of similar phosphorus content are precisely pipetted into silver crucibles. To each is added 0.2 ml. of 50-per-cent KOH, the contents are evaporated to dryness, treated with 1.0 ml. of 25-per-cent KNO_3, and stirred with a rod. Then 1.0 ml. of water is added, washing the rod. The contents are warmed, stirred again, the rod washed with a few drops of water, and the contents re-evaporated to dryness, and fused with approximately 0.9 gram of KOH (about eight pellets).

A or B. In either case the melt is cooled and transferred to a 50-ml. Pyrex beaker, as much as practicable directly, followed by hot distilled water washings of crucible and lid. The contents are made *acid* to Congo red paper (20) by addition of the nitric-sulfuric acid mixture of Niederl and Niederl (1), and evaporated almost to dryness. The solution is passed through a sintered-glass filter into a tube marked at 15-ml. volume followed by two one-milliliter washings of the nitric-sulfuric acid mixture and several of water. The solution is made up to 15 ml. with water, and heated to 81°–85°C. in a water bath. The determination is completed according to Niederl and Niederl, with two exceptions. The final ether washing is omitted, and after weighing the filter and precipitate, the latter is dissolved from the filter with 1:5 dilution of ammonium hydroxide, followed by washings of ammonium nitrate, ethyl alcohol, and acetone as before (1). The filter is weighed finally and the weight difference used to calculate phosphorus content by multiplying by an empirical factor (0.01454).

Lipids, Method of Extraction.—Lipids are extracted from plasma or serum by the method of Bloor (21).

Cholesterol, Free and Total (Gravimetric Method).—Prepare the sample for analysis and precipitate cholesterol as digitonide according to Yasuda (22). Dry and weigh the precipitate according to the procedure of Osato and Heki (23).

Cholesterol, Free and Total (Oxidative Method).—Yasuda (22).

Iodine Number of Lipids.—Yasuda (24).

Fatty Acids.—Street (25).

Acetyl Groups.—The method of Elek and Harte (26) is used, modified as follows:

Following hydrolysis of the sample by *p*-toluene sulfonic acid, the liberated acetic acid is distilled under reduced pressure into a flask containing water and is subsequently titrated with *N*/70 NaOH.

***p*-Aminobenzoic Acid** (**Free or Conjugated**), **and *p*-Nitrobenzoic Acid.**—Eckert (27).

Calcium, in Culture Media.—(28-a).

Calcium, in Serum.—Holt and Callow (29).

Chlorides.—For chloride determination in solutions, the titration technic of McKittrick and Schmidt (30) is preferred. In blood serum, other body fluids, and tissues the material is treated by the method of Van Slyke and Sendroy as modified by Wilson and Ball (2-c, 31). However, it may be preferable to titrate finally by the method of McKittrick and Schmidt.

Magnesium, in Culture Media.—(28-b).

Mercury.—Eckert (32).

Reducing Substances Affecting Ferricyanide Reagent (**33–40**).—**Total** (***G***), **nonfermentable** (***r***) **fermentable** (***g* = *G* − *r***).—Broth or infusion is first treated to remove proteins by means of a modification (33) of the defecation procedure of Johnson and Renfrew (34) as follows:

Defecation.—Into a 125-ml. Erlenmeyer flask measure precisely 40.0 ml. of sample (broth or infusion), 20.0 ml. of 10-per-cent sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), and, with agitation, 20.0 ml. of approximately N H_2SO_4. Centrifuge 10 minutes at 2000 r.p.m. in two 50-ml. tubes and filter through a suitable filter-paper previously washed with water and rinsed by filtering and discarding two small portions of supernatant liquor. Call the filtrate F.

Make *solution A* from 40.0 ml. of F with or without a fine white suspended precipitate plus 60.0 ml. of approximately $N/18$ NaOH. A clear solution should result. The amount of precipitate transferred in F does not seem to have appreciable influence on the results, and the filtrate F and solution A are satisfactory for use throughout the day's work, at least five hours.

The next part of the analysis is essentially the procedure of Ionesco-Matiu and Vitner (35). Water redistilled in glass from alkaline $KMnO_4$ will be called simply redistilled water in the description of this technic and the titration with ceric sulfate which is essentially according to Miller and Van Slyke (36). The procedures are applicable to solution A above, or to *solution B*, which is A minus the fermentable substances removed by washed yeast by a technic described later (p. 120).

Ferricyanide Reagent (**Ionesco-Matiu and Vitner**) (**35**).—To a solution containing approximately 23 grams of KOH (a suitable portion of a strong solution of known content) add 23 grams of $K_3Fe(CN)_6$ of reagent grade and water, preferably *redistilled*, to dissolve and make one liter. Stored in a brown glass-stoppered bottle, the reagent is stable for many months and blank values are constant if redistilled water is used.

Ferricyanide Reduction.—Of the prepared solution (A or B) precisely pipette a convenient volume V ml. into a 50-ml. lipless round-bottomed centrifuge tube in which the rest of the analysis is performed. Add approximately $(6 - V)$ ml. of water, 1 ml. of approximately $2\,N$ NaOH, 1 ml. of ferricyanide reagent, and 10 ml. of water. Mix well. Immerse the tube to within 4 cm. of its top for precisely 12 minutes in water which is kept boiling vigorously enough to be well circulated; then cool without delay in running tap water for at least 3 minutes.

Blank Determinations.—Blanks should be run essentially as above with no sample used; that is, $V = 0$.

Titration.—The titration should be performed within one hour after the reduction, as in the method of Miller and Van Slyke (36). Just before titration of each tube, add 1 ml. of approximately $18\,N$ H_2SO_4. Then add 7 drops of 0.1 per cent Setopaline C (or other suitable indicator) and enough standard $Ce(SO_4)_2$ to produce the characteristic golden brown end point. The color fades on standing a few minutes, mostly as a result of slow oxidation of substances other than the ferrocyanide by the slight excess of $Ce(SO_4)_2$ required to give the end point. It is usually preferable to add the last four drops of indicator just before the end point is reached. It is convenient to use a 0.005508 N ceric sulfate standard to save space in the tube. This is twice the strength used by Miller and Van Slyke, each milliliter being equivalent to 0.2 mg. glucose. Let T be the titer in milliliters with 0.005508 N $Ce(SO_4)_2$ and b the corresponding blank value. Then the glucose equivalent (X) in grams per liter is given by $X = (T - b)/V$. G is the value of X obtained for solution A and r is the value for solution B.

Standard 0.1377 *N* $Ce(SO_4)_2$.—A stock ceric sulfate solution is made, standardized against a high-grade preparation of Mohr's salt $FeSO_4(NH_4)_2SO_4 \cdot 7H_2O$, and most of this is precisely diluted to 0.1377 N concentration as described by Miller and Van Slyke (36) except that redistilled water is used. This is stable for about a year. However, it is readily restandardized, and may be reinforced by additions of small reserved amounts of the strong original solution, if required, and so used years later.

Standard 0.005508 *N* $Ce(SO_4)_2$.—In a precisely calibrated 250-ml. volumetric flask put 12 to 13 ml. of approximately $18\,N$ H_2SO_4, precisely 10.00 ml. of 0.1377 N $Ce(SO_4)_2$, and dilute with *redistilled* water to volume. Store in a dark, glass-stoppered bottle. If water redistilled in glass from alkaline $KMnO_4$ is used the dilute standard is stable for two weeks within 1 part per thousand. Otherwise there may be a loss of strength amounting to 0.5 per cent in six hours at room temperature or 0.6 per cent in a week in a refrigerator (36).

Nonfermentable Reducing Substances (*r*).—Fermentable reducing substances are removed from a portion of A by treatment for 15–20 minutes with washed yeast (37–39); and, after centrifugation, the supernatant B is used for the determination of residual reducing substances (*r* in glucose equivalent) as indicated above.

The technic is essentially the same as that of Peters and Van Slyke (2-d), except that the portion of resuspended five-times-washed yeast, transferred to a 50-ml. centrifuge tube, centrifuged, and left after decanting the supernatant (38) is not wiped with rolls of filter paper, but instead the surface of the packed yeast is twice washed gently with small portions of the solution A. These washings are discarded, and a volume of A added which is about 4 times that of the packed yeast cells; the yeast is suspended by stirring with a rod, left 15–20 minutes at room temperature, centrifuged, and the supernatant liquor is decanted. This is *solution B*. Recent modifications of the yeast fermentation-technic by Stark and Somogyi (39) permit removal of glucose or glucose and maltose by appropriate selection of pH.

Fermentable Reducing Substances.—As indicated above, fermentable reducing substances are given by $g = G - r$, in milligrams of glucose equivalent. Glucose and maltose in the same preparation may be estimated by difference as indicated above (39) or gasometrically (p. 109).

NOTE: An investigation of the influence of various substances upon the reducing action of glucose countered by a ferricyanide reagent or by a copper reagent has been reported recently (40).

Sulfonamides in Blood and Urine.—The methods as developed by Marshall and associates (41–43) are used for determination of sulfanilamide and sulfapyridine in blood or urine.

Tryptophan.—Eckert (44).

Micro-Kjeldahl Nitrogen.—Samples containing about 0.5 to 1.5 mg. N are usually employed. One to 1.25 ml. of Campbell and Hanna's acid mixture (45) is used in digestion, supplemented by use of 2 drops of perhydrol in the usual way. Other digestion technics and the general principle of the method are given by Peters and Van Slyke (2-e).

The principle of the steam distillation technic as there given is employed in an all-glass apparatus. The distilled ammonia is collected in 10 ml. of 2-per-cent boric acid containing 4 ml. of 0.1-per-cent methyl red in 95-per-cent alcohol per liter, and titrated with $N/70$ H_2SO_4, using color standards diluted to the same final volume and containing boric acid, indicator, and enough H_2SO_4 or NaOH to represent the end point and over or under titration, requiring respectively -0.06, -0.03, 0.03, and 0.06 ml. correction to the titer (46).

Transfer Rod, Dead-Line Knob (47).—For transfer of precipitates (for example, from centrifuge tubes to Kjeldahl flasks) a rod with handle and dead-line knobs has been devised. The rod is made from either pyrex- or flint-glass stocks of 3 mm. and 8 mm. diameter. The larger provides a handle about 6 cm. long with a knob about 13 to 15 mm. in diameter acting as a hilt. This is formed by rotation in a blast lamp flame and slight compression. Beyond this the rod is drawn down to about 3 mm. diameter and sealed to the rod of that diameter with compression again to form a small knob about 6 mm. in diameter. The small rod is cut to convenient length (usually 7 to 12 cm.). The end is tapered slightly for about 2 cm. down to a diameter 1 to 2 mm. using a grinding wheel. The ends are washed, dried, and fire polished. The tip must be polished with care to avoid spoiling the taper which helps in transfers from tubes. The smaller knob tends to prevent travel of washings, etc., above that point; the larger knob is a dead line below which the operator's hands should not pass. The knobs also serve to permit laying the rod on a bench temporarily without test material being lost—the large knob holds the smaller rod clear, the small knob limits run-back of material beyond that point.

SPECIFIC GRAVITY METHODS

Several methods have been developed for the rapid determination of the specific gravity of the blood and applied to estimation of the protein concentration of plasma or serum. Two of these methods have been used in the laboratory for the determination of protein in various animal sera for therapeutic use and in the human sera submitted for routine serologic tests.

Densiometer.—Barbour and Hamilton (48) have described a technic which involves the timing of the fall of a drop containing precisely 0.01 ml. of serum through a definite distance in a fluid nonmiscible with the serum, and comparing the result with the dropping time of an equal amount of standard K_2SO_4 solution of known density. The specific gravity of the serum is obtained with the aid of a nomogram which takes into account the temperature, dropping time of the serum droplet, and that of the standard K_2SO_4 solution. This yields a term which is added to the density of the standard at the operating temperature to give the serum density. The nomogram does not compensate for differences in temperature; and, accordingly, the standard K_2SO_4 solution should be retested if changes in temperature occur during operation.

Copper Sulfate Method (49).—A simple precision technic for determining the specific gravity of whole blood, plasma, or serum is based on the fact that when the protein sol is dropped into a solution of copper sulfate of known gravity it is normally encased in a sac of copper proteinate and the gravity of this drop is not changed for about fifteen seconds. The rise or fall of the drop during this interval indicates whether its density is greater or less than that of the copper solution. Three or four drops of blood, plasma, or serum, the size of which need not be accurately measured, are sufficient for a determination. The gravity of the protein sol can be determined to ±0.0002 and temperature controls are not necessary. In work with antipneumococcus rabbit serum (50) the protein was estimated by the same formula used for normal human plasma.

Total Protein Estimated from Specific Gravity.—The protein concentration of the serum is estimated from the specific gravity (G, determined by either method) by the formula

$$X = K(G - D)$$

where K and D are constants. D may be considered as approximately the specific gravity the serum would have if freed from protein. Several values for K and D have been suggested. Approximately, $K = 376$, and $D = 1.007$, as proposed by Phillips and others (49) for normal human plasma, have been found satisfactory in this laboratory.

Globulin.—Another example of the use of the falling-drop technic is the determination of the amount of globulin precipitated from serum. The precipitates are dissolved in a buffered salt solution and the dropping time of the solution is determined and compared with that of the buffered salt solution containing no protein. The apparent density difference is then estimated by use of the nomogram and this difference is multiplied directly by the factor $K = 376$ to estimate the percentage of protein in the solution, and thence the amount of protein dissolved. The results agree approximately with those obtained by the micro-Kjeldahl technic with solutions containing about 0.5-per-cent protein.

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CHAPTER 10

VOLUMETRIC CALIBRATION AND MICROVOLUMETRIC MEASUREMENT

VOLUMETRIC CALIBRATION

Unless otherwise specified, the methods employed for calibration of volumetric apparatus are essentially the same as given by Peters and Van Slyke (1-a). However, a large portion of such work is done by means of an apparatus (2, 3) designed in this laboratory for rapid precise calibration of pipettes for delivery between marks or for blow-out delivery. The system has been extended to calibration of pipettes *to contain* and approximately in simulation of simple *drainage* delivery, as well as to calibration of small volumetric flasks (100 ml. or less), microburettes, Van Slyke-Neill or Van Slyke-Folch chambers (4), and other types of apparatus (2, 5).

Pipettes.—A salient feature of the system is that new marks may be precisely located directly rather than indirectly through successive approximation as in other methods.

In this way, ten 0.5-ml. Ostwald pipettes for *blow-out* delivery were to be re-marked for use in special work. The new marks were located by use of the calibration apparatus and a new ring was etched on each (with an arrow pointing to this corrected location as is customary). The pipettes were then recalibrated with two observations each by two persons. The mean calibration values obtained (in ml. 10^{-4}) were as follows: 5003, 5030, 5008, 4984, 4989, 4998, 4983, 5008, 4996, and 4999. In the forty observations, deviations (*d*) from the corresponding mean values (given above) never exceeded 0.0008 ml. and the standard deviation estimate (based on the 40 observations with 30 *degrees of freedom* (6) was less than 0.00039 ml.

The main features of the apparatus are indicated in Fig. 17 showing the modified form (3). Stopcocks A′, B′, and D are usually set part open to control flow rate; the others are used as open or shut gates. Mercury, indicated by solid black in the diagram, fills the reservoir, standard pipette, connecting tubes beneath, and part of W above which is water. Mercury seals are at Q and G. S is the standard pipette in which mercury is the only liquid used, which (as it does not wet glass) causes no drainage errors in the standard. Entrapment of air in the vessels under the mercury level is avoided by careful filling with the aid of a good vacuum above. Air entrapment in the water regions is less important but is carefully minimized also, the water being made to rise in the pipette P ready for calibration. This is drained to the lower mark L (which may be at the tip as in *blow-out* calibration) in a given drainage time (*D.T.*) controlled by setting A′ and D in trial drainage. It is preferable to use D thus for flow control (3) rather than C as in the original technic (2). After drainage as required, the pipette P is refilled by running mercury from S controlled by stopcock B until the required volume *V* is delivered, returning the water level in P to U or else a new upper mark to be set on the pipette P if required. Details of technics are given in the original article including the use in some cases of M for fine adjustments of level in P, prior to the refilling, by producing a tambour effect on the rubber stopper at T. Pipettes may be calibrated *to contain* by use of very slow drainage approximating complete drainage as a limit, or by only partly wetting the pipette by inserting it (after drying) through the flooded rubber stopper at T with careful removal of the remaining water in slow drainage ($D.T. =$ "∞").

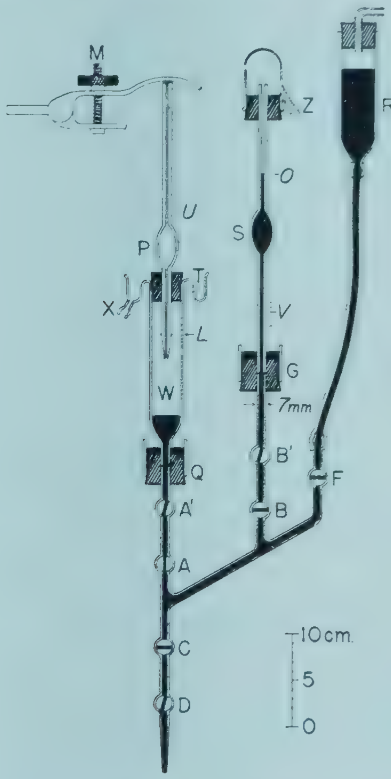

Fig. 17. Modified Calibration Apparatus

Flasks.—In a similar manner, dry volumetric flasks may be calibrated. A delivery tube is inserted at T instead of the pipette P. It is in the form of an inverted U ending in a fine tip, completely filled with water. From this, water is forced into the flask quantitatively by delivery of mercury from the standard pipette as before. The delivery tip should be touched to a water surface in a beaker just before the delivery and to the water surface in the flask at the finish.

Microburettes.—A modified vessel W as shown in figure 18, but preferably of as small capacity as practicable (3), is used for calibrations by direct flow from a burette to the standard pipette, which in this case is inverted (O below and V-scale above). Actual use of the S-pipette inverted is permissible if its arms are of essentially the same diameter, otherwise a correction would be required for difference in volume of meniscus depression. In this way successive small portions of the microburette may be calibrated, preferably corrected by adjustment *pro rata* from an over-all calibration under given continuous drainage conditions. Thus calibration may be reinterpreted readily in terms of different drainage rates without repetition of calibration of individual subintervals.

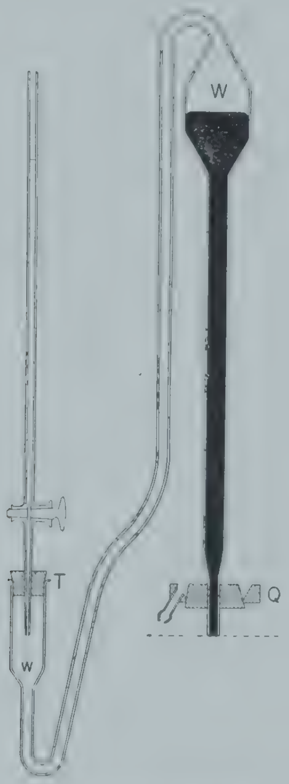

FIG. 18. MODIFIED VESSEL *W* FOR CALIBRATION BY DIRECT FLOW, ALL ONE PIECE FROM *Q* TO *T*

Reproduced by permission from Industrial and Engineering Chemistry (Ind. and Eng. Chem., Analyt. ed., 1942, **14**, 268).

Van Slyke-Neill Chambers.—The chamber of the Van Slyke-Neill manometric apparatus or modified forms (4) may be calibrated by proceeding as above for microburettes, first filling the chamber and part of the cup above it with water forced upward through the stopcock, but keeping the other bore of the stopcock and the adjoining delivery tube dry. The stopcock is turned to open this dry path just before opening B to permit drainage at a given rate (controlled by A′ and B′ adjusted in preliminary trials) and measurement of the volume in an appropriate S-pipette.

The various marks (nominally 0.5, 2, 10, and 50 ml. on the Van Slyke-Folch modification (4) are calibrated thus with a return to zero before each measurement—filling the chamber as before. The required standard pipette (having the same nominal value) is set at G in the inverted position, several observations are taken and the mean used in each case. Usually different observers deviate by only a few thousandths of a milliliter in individual observations. Of course, the drainage time should be properly controlled and recorded as an essential part of the data.

Gas Burettes.—Gas burettes having a 2-way stopcock on top may be calibrated in a similar manner; for example, the burette for the Van Slyke gas volumetric apparatus or that for the amino acid determination by nitrous acid reaction.

Glass Syringes.—Apparatus for ejection of given volumes of solution, such as glass syringes, may be calibrated by use with the same apparatus of another modification of the vessel W (5). This, all one piece of glass, consists of a vertical connecting tube about 45 cm. in length at the top of which is a bulb of convenient size surmounted by a cup with a short, small-bore connection upon which is etched a horizontal ring U. This vessel is joined at Q to the calibration apparatus (figure 17) or to a 3-stopcock modification of this as sometimes used for a microburette system discussed below (figure 19). The chamber W is partly filled with mercury with water above, which has its upper level set at the ring U. A suitable S-pipette is used in the inverted position, at G as in burette calibration, with the mercury level in S at O. The syringe with needle attached is filled with water as usual to nominally the same value as S. Slightly less than this volume is allowed to pass from W and flow into S. Without undue wetting of the cup on top of W, the syringe is then discharged into W as directly as possible, and liquid further withdrawn to S till the water level is returned to U in W. The volume is then read on the V-scale.

The system may be used for measurement in this manner of the sum of a number of successive deliveries of a smaller volume (nominally a submultiple of the volume of S) and thus calibrations of a syringe over-all and in simple subranges (by repeated delivery) can be performed without changing the S-pipette.

Standardizations and Check Calibrations by Mercury Weighing.—Calibrations may be made on S-pipettes or mercury microburettes inserted in the same position (at G in figure 17) or apparatus inserted as usual at T, by carefully collecting and weighing an equal volume of mercury withdrawn from the tip below D into a weighing bottle, the tip being touched to a mercury surface in a beaker before and to that in the weighing bottle after the delivery, and the temperature noted. In such calibrations, flow should be controlled in the same way as in use of the apparatus, for example, at B in calibration of S-pipettes (2) or mercury microburettes, at C for apparatus placed at T. Of course, the adjustment at M should not be used in this case except for the setting at U. From tables (1) the volume delivered is calculated. Deviations from nominal values are usually found in careful recalibrations. Correction tables should be kept handy and applied as required in precise use of the standardized apparatus; as in use of S-pipettes to calibrate other apparatus.

Volumetric Calibration of Mercury Microburettes.—Mercury microburettes, such as are used in the system described below, which have been carefully calibrated in the manner given above, but in the position at Q (without the use of a chamber W) may be used to calibrate other mercury microburettes placed at G. In this way the V-scales of S-pipettes may be calibrated, but the over-all calibration from O to a principal reference mark on V should be made directly as in the preceding section, and the results combined to give the correction table required in use.

Calibration of Ostwald-Van Slyke Pipettes of Small Volume. A standardized mercury microburette (for example, 0.25 ml. subdivided in 0.002-ml. intervals, total scale length about 280 mm.) may be used in place of an S-pipette to calibrate pipettes of small capacity.

Thus new marks were set on six 0.2-ml. Ostwald-Van Slyke pipettes (designed to deliver between marks). Subsequent recalibration after etching gave mean volumes in 10^{-5} ml. as follows: 20085, 20026, 20032, 20004, 20018 and 20001. Each was the mean of eight observations, half by each of two observers. None of the 48 observations deviated from the corresponding mean by more than 0.00042 ml.; the standard deviation estimate for individual observations (48 with 42 degrees of freedom (6)) was 0.00016 ml.

Influence of Drainage Rate.—What is to be tolerated depends on the circumstances; but for precise analytical work, pipettes usually should be calibrated for drainage in not less than 15 seconds for any volume, 20 seconds for 10 ml., 30 seconds for 25 ml., and 40 seconds for 100 ml.

Three 1-ml. Mohr pipettes (with the graduated portions about 20 cm. long) that had been recalibrated for delivery in 15 seconds, were found to give for indicated delivery times in seconds the following mean volumes expressed in per cent of the nominal value: 2, *97.9*; 3, *98.5*; 4, *98.9*; 5, *99.2*; 7, *99.5*; 11, *99.8*; 15, (*100.0*); 20, *100.1*; 50, *100.4*; 100, *100.5*; 500, *100.7*. This indicates the relative errors that may result from too rapid drainage.

Tolerances in Pipette Design.—For precise work, further specifications given by Peters and Van Slyke (1-b) should be adopted also, particularly that the inner diameter of the stem should be such that a 1-mm. length contains less than one-thousandth of the nominal volume of the pipette. In general a 0.5-mm. error in reading the pipette should lead to error well within usual tolerances. Instead of using a fine opening at the tip to control delivery, it is often preferable (1-b) in biochemical work to have larger openings and retard delivery by manual control.

MICROVOLUMETRIC MEASUREMENT

To meet a need for a means of safe handling and precise delivery of small amounts of dangerous material or special reagents, a microburette system (7) was developed. Where the precision is adequate, a safety pipette filler (8) may be used for measurement of dangerous material with ordinary pipettes, see pages 8 and 106. However, for micromeasurements such as are required where it is preferable to use small amounts of strong reagents for the sake of their stability, or to avoid undue volume increase on their addition to a medium to be adjusted, or in various micromethods, the microburette system is preferable. Reagents are placed in storage-delivery vessels W of various types which may be attached to a control device essentially the same as used for volumetric calibration or a 3-stopcock modification as indicated in figure 19, which is simpler but less convenient. Capillary tubing and stopcocks of 1- or 2-mm. bore may be used to advantage in the case of micromeasurements, to reduce total volume in the system, with a consequent reduction of errors due to temperature variations, and to facilitate control of flow rates. The apparatus as shown in figure 19 has been used in studies of serologic reactions of blood serum to make micro-adjustments of pH without undue increase in volume, using one-third normal NaOH. Convenient microburettes for the mercury measurement are made from capillary tubing with outside diameter about 7 mm. (like the stems of the S-pipettes) graduated to cover a range of 0.1 ml. in about 175 mm. subdivided in 0.001-ml. intervals, or 0.25 ml. in about 280 mm. subdivided in 0.002-ml. intervals. Beyond the graduated portion the burette should extend about 10 cm. on each end. For larger measurements larger burettes are useful, and S-pipettes may be used conveniently also where the same volume is to be delivered many times in succession.

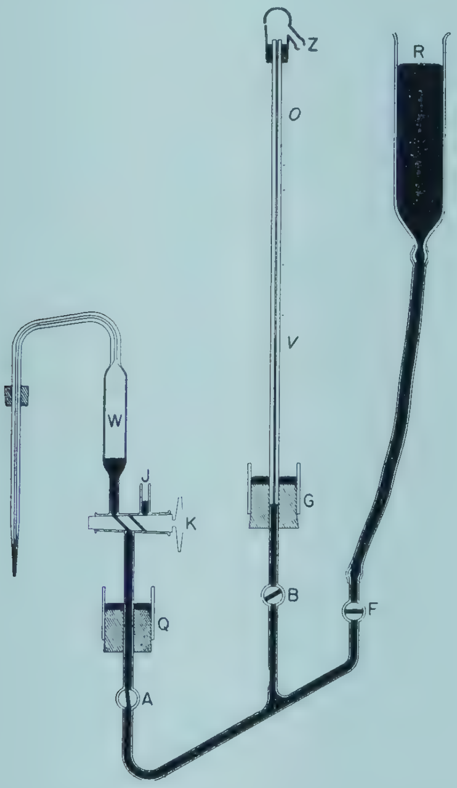

FIG. 19. MICROBURETTE SYSTEM WITH SIMPLE STORAGE-DELIVERY VESSEL AND ABBREVIATED STOPCOCK SYSTEM

Storage-Delivery Vessels.—The storage-delivery vessels W are provided with a 2-way stopcock K near the base, with the *trunk* below and two *branches* above, one of the latter leading to a bulb in W and the other terminating in a stump J. The vessel is attached at Q as is the burette at G by insertion of the trunk end through a pool of mercury in the cup, with the stopcock turned to J which is used as a vent. Some mercury is run up into J with care to avoid entrapment of air, and the stopcock rotated 180°. The reverse process is used in detaching the vessel.

The simplest form of storage-delivery vessel (shown in figure 19) consists of a bulb above K like that of a pipette with a capillary delivery tube at the top turned down in an inverted U or *crook* ending in a long vertical delivery tip. To fill the vessel, it is attached to the control system, filled with mercury from the reservoir R, and then reagent is drawn in through the tip, mercury being removed to the lowered reservoir or through stopcocks C and D below if the seven stopcock system is used as shown in figure 17.

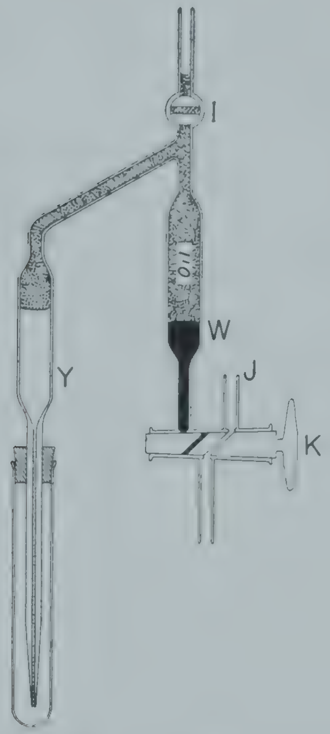

FIG. 20. STORAGE-DELIVERY VESSEL WITH OIL INTERMEDIATE

Another form of storage-delivery vessel is shown in figure 20, where oil separates reagent from mercury. It is filled by first allowing mercury to rise as high as practicable in W without risk of overflow into the descending tube leading to Y, then drawing oil into the delivery tip and the rest of the vessel by removing air through stopcock I at the top, closing I and finally drawing reagent in through the tip by withdrawing mercury from W (either to the reservoir or otherwise) as in the case of the simpler storage-delivery vessel. Other vessels may be constructed with small capacity and a gas space to separate mercury and reagent.

In use for delivery of reagent, the stopcock K is left wide open to the chamber W in all cases, A′ (or A in the simpler model) used to control flow-rate and B open or shut—usually rotated 180° several times towards the end of the delivery. Measurements can be made easily within a tolerance of 0.0002 ml. A glass bell dust shield may be attached by the rubber stopper shown on the delivery tip if required.

When not in use, the delivery tip may be enclosed in a shielding tube as shown in figure 20. If mercury is to be allowed in contact with the reagent but air excluded, some mercury may be placed in this tube to seal the tip. The vessel may be detached at Q in the manner described above, and hung by the crook of the delivery tube over a horizontal bar. Thus several reagents may be kept ready for immediate use.

The W-vessels may be sterilized by autoclaving before or after use as required; but, if so, a stopcock lubricant should be used which will not become too fluid with increased temperature (for example, Nevastane X, Dow-Corning stopcock grease, etc.), and account should be taken of the fact that if stopcock bores are left full of mercury the thermal expansion will force some between the plug and jacket of the stopcock necessitating cleaning and regreasing.

DISCUSSION

Standard taper stopcocks are used throughout for both the calibration and the microburette system, and pyrex glass is preferable except for a minor consideration that if apparatus to be calibrated is made of the same glass as the S-pipette (or burette) used, then no correction is required for difference in thermal expansion (2). At least one of the stopcocks (A, B, C, F) should be open at all times except briefly to permit deliberate operation, and when the apparatus is left idle F should be the stopcock left open and the reservoir R should be placed so that its mercury level is only slightly above F. During such idleness air enters the rubber tubing slowly; and, before use of the apparatus, the reservoir should be raised again and the rubber tube gently manipulated to expel this air through the reservoir.

A mixture, about equal parts at room temperature, of the regular and the newer high vacuum type Dow-Corning stopcock greases (both silicone products) is an excellent lubricant and seal for stopcocks and ground joints in general, except where contraindicated as in combustion apparatus (5).

The difference Δp in pressure just below the S-pipette or microburette at G when filled with mercury to the upper mark O or only to a lower mark V is usually less than 24 cm. of mercury, the distance between O and V. This results through slight expansion at G with the increase in pressure Δp in a decrease in volume ΔV in the S-pipette or microburette of approximately 0.001 ml. or less, and this is compensated by the suggested method of standardization (2). However, it is well to determine this depression value at the time of standardization and check it each time the standard is reset in position ready for use. This may be done readily with the mercury level at V by attaching a line at the top of the standard pipette or microburette leading from a bottle, enclosed in a casing for safety and provided with an open-type mercury manometer for pressure reading; the required increase of pressure is developed with a small hand pump or atomizer bulb. A 2-way stopcock or two single-way stopcocks should be used, one leading to the pump and one to the atmosphere to provide a ready release of pressure. Thus ΔV is easily evaluated.

This influence can be sensibly completely eliminated by use of ground-glass joints at G between the apparatus and the S-pipette or microburette. Standard taper (10/30) joints with glass hooks are suitable. They are lightly greased the same as stopcocks. The inner joint should be below, sealed to the tube leading upwards from stopcock B′, with a collar in place of the well at G (merely to catch mercury dropped when the joint is later disconnected). Outer joints should be sealed on the bottoms of microburettes or S-pipettes and also on the tops of the latter if they are to be used occasionally in the inverted position. Although the joints should be made of the same glass as the apparatus to which they are sealed, pyrex for the stopcock-control apparatus, it is still permissible to employ standards of the same glass as used in the apparatus undergoing calibration, if this refinement is desired to eliminate corrections for thermal expansion. When apparatus is being filled with mercury through such a joint, a vacuum must be applied above to prevent entrapment of air. The joint alone may be used as support of microburettes and small S-pipettes. Otherwise, some other support of the main load must be provided without unnecessary strain on the glass; e.g., a disk around the stem supporting the bulb and resting upon a ring clamp assembly permitting horizontal shift to avoid a breaking strain.

However, the risk of breakage from such strains, due to extra rigidity of the apparatus when standard taper ground joints are used instead of the rubber joint originally employed, may be eliminated by insertion of a semiball and socket joint between the S-pipette or microburette and stopcock B′. It is convenient to have the standard taper joints (10/30) as before, but to use Pyrex adapters also, one with S.T. outer joint (10/30) and inner ball joint (18/7), the other with outer ball joint (18/7) and inner S.T. joint (10/30). These may be obtained from the Scientific Glass Apparatus Company, Bloomfield, N. J.

A good flexible clamp for the semiball joint may be made from a 5 to 6 cm. length of rubber tubing having a bore about 1/4 inch and 1/16 inch wall. About 1 cm. from each end three symmetrically placed holes (3 to

4 mm. diameter) are bored and longitudinal slits are cut between pairs of holes. This leaves a 1-cm. collar at each end and three joining strips. The collars are slipped over the adapters, the joint greased and set, and the collar on the one side pulled down the stem so it may be wired to the outer standard taper joint and produce tension enough to hold the ball and socket joint firmly together.

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CHAPTER 11

THE SPECTROSCOPIC LABORATORIES

INTRODUCTION

The spectroscopic laboratory was established more than a decade ago to serve as an aid in the elucidation of immunologic and biologic problems, to supplement other methods of analysis, and to aid in the establishment of standards. Most of the phenomena of immunology are physicochemical in nature and study of them leads to fields that may seem to have little connection with the question of immunity. Spectroscopic methods offer means of discriminating between substances of different chemical natures and assist in the solution of many biochemical problems.

Obviously it has not been possible in this period of time to develop and standardize all of the methods and applications to the work in the various services of the laboratory envisioned in the beginning (1) and, since then, greatly extended. Considerable progress has been made, however, both in the technical work and in special investigations (2–12). These studies have yielded information concerning substances occurring in laboratory products which has been helpful in their preparation. The substances that have been investigated are those that in plant and animal organisms have to do with metabolic activities, nutrition, respiration, and antihemorrhagic, enzymic, and catalytic activities.

Absorption spectrophotometry was the first of the physical methods to be adopted since, in certain instances, it is the only means of identifying a substance when its chemical nature is unknown. This method of analysis usually does not disrupt the molecule, measures small concentrations, requires little material, and is accurate. Moreover, spectrophotometric data may be expected to yield information about the structure and configuration of the molecule, chemical bonds, effects of substituent groups, moments of inertia, and oxidation and reduction in the study of enzymes, and also to give an indication, in certain cases, of changes in the transition of the total internal molecular energy that comes from the combined motions of its constituent electrons, from the vibrations of its constituent nuclei, and from the rotation of the molecule as a whole. Since these various energy states are of the order of a few electron volts, a few tenths of a volt, and 1/100 volt above the ground state, respectively, manifestation of changes in electronic energy may be sought in the visible and ultraviolet regions of the spectrum, and those of nuclear energy (vibration and rotation), in the infrared region. Therefore, the spectroscopic instruments acquired for the application of this method cover the ultraviolet, visible, and infrared spectral range from 2000–17,000 Angstroms and consist of a Hilger quartz spectrograph E316 with Spekker photometer, a Hilger-Nutting spectrophotometer with camera attachment for the visual range, a Hilger infrared spectrometer, a microspectroscope, and a Pulfrich photometer. A Zeiss photoelectric recording photometer and a Hilger measuring micrometer with double verniers reading to 1/10,000 mm. are used to evaluate the photographic plates made with the various instruments. A list of spectroscopic equipment is given on p. 141.

All of the instruments assembled are suitable for study not only in absorption spectroscopy but also in the vast field of emission spectroscopy, and for investigation of fluorescence; some, for studies of Raman spectra. Thus, they are adapted to problems in research work requiring high precision in the realm of pure physics, as well as in immunology and biochemistry. However, there are now available automatic recording instruments of a standardized type for studies in spectrophotometry which supersede in convenience and rapidity of operation these nonrecording instruments.

SPECTROSCOPIC AND OTHER PHYSICAL METHODS

Absorption Spectroscopy.—The accuracy of the spectroscopic procedure was tested in the visible region by a comparison of absorption curves for copper sulfate ($CuSO_4 \cdot 5H_2O$) and for potassium chromate (K_2CrO_4) solutions in different cell depths, with the published curves that are standards of reference for these substances (13–15). In the ultraviolet region the procedure was tested by a comparison with curves for potassium chromate (16, 17).

The method used at the present time for plotting absorption spectrum curves for publication is as follows: The wave lengths in Angstroms, A, and frequencies in fresnels are plotted along the abscissae at the bottom of the chart and the wave numbers in $cm.^{-1}$ at the top of the chart, against absorption data expressed as extinction coefficients, specific extinction coefficients, or molecular extinction coefficients, as the case may be, along the ordinates.

Fluorescence Spectroscopy.—The method of fluorescence spectroscopy is employed to determine the emission band maxima and minima of substances that exhibit fluorescence when irradiated with ultraviolet light. The fluorescence spectra of certain substances may be as characteristic as their absorption spectra.

Fluorescence spectrograms are recorded with either the quartz spectrograph or the Hilger-Nutting spectrophotometer, depending upon the wave length positions of the bands. The spectrograms are then evaluated by means of the Zeiss photoelectric recording photometer in the same manner as those for absorption spectra. The sources used to excite fluorescence are usually either the Hanovia Analytic Quartz Lamp or the General Electric B-H4 mercury lamp. The Cooper-Hewitt Lab-Arc with suitable filters has also been used.

Chromatographic Analysis.—The chromatographic method of analysis, inaugurated by Tswett (18) and revived by R. Kuhn (19, 20), has proved to be a valuable method for the isolation and purification of substances from mixtures for spectroscopic study. A micromethod of ultrachromatographic analysis—that is, chromatography combined with fluorescence analyses—has been developed (9) that requires only a few drops of solution and is useful for a rapid preliminary survey before preparing solutions by chromatographic analysis for the purpose of spectroscopic investigations.

Crystallography.—In addition to spectroscopic analysis, it is sometimes necessary, in order to establish the identity or nonidentity of two substances, as for instance, certain polyenes, to resort to analysis with "mixed chromatograms" or to make a study of crystal forms and aggregates and of the determination of melting points. Hence the application of some of the methods of crystallography is under investigation. The Kofler hot stage is used for the determination of melting points (corrected).

Emission Spectroscopy.—The methods of absorption spectrophotometry and fluorescence spectroscopy are those now employed for the most part in the use of the instruments since biologic substances that require delicate treatment and that are sometimes easily decomposed by chemical methods are under investigation. Treatment of such material by electric arc or spark discharge (as in studies of emission spectroscopy) is disruptive. Nevertheless, the emission method is of great value in detecting the presence of very small quantities of metallic and certain nonmetallic elements in organic materials when chemical methods fail.

Emission spectroscopy covers a wide field and involves a highly specialized technic that is very different from that employed in the practice of absorption spectrophotometry. However, provision has been made by the purchase of needed accessories such as certain pure metallic electrodes, "raies ultimes" powder, and charts for further development of emission spectroscopy, for example, detection of metallic elements in organic materials. New problems arise in dealing with the different elements when occurring in different materials. Hence the method used in one case is not applicable to all.

Mercury usually is detectable by ordinary arc or spark spectrum analysis, but when it is diffused in a small quantity throughout a mass of organic substance, a special technic is required. Small quantities of mercury in solutions of organic material were detected by means of emission spectroscopy combined with electroanalysis when chemical methods failed. The emission spectra may be obtained by passing an electric spark through a solution of the material to be tested in a Hitchen sparking tube[1] or by passing a spark in air between a gold electrode and a copper electrode that has been used as a cathode in electrolyzing the solution. The electrolytic deposition of the mercury in the solution on a suitable electrode is a simple way of concentrating mercury that is present in very minute quantities for spectrographic study. The spectra may be recorded with the quartz spectrograph on Kodak 33 photographic plates, on special spectroscopic plates, or on sensitized plates, depending on the requirement of the problem.

Nephelometric Standardization of Glass Standards of Turbidity.—Glass standards of turbidity have been substituted for barium sulfate standards in the laboratory. The stock suspensions cannot be prepared to have a definite initial turbidity. Hence, it is essential to have a physical method for their characterization so that they may be replaced satisfactorily from time to time.

The turbidities of stock suspensions are determined with a Klett-Summerson (KS) photoelectric colorimeter and standards are prepared by dilution and by calibration with this instrument (p. 26).

A more precise physical method for the characterization of these suspensions is available; but it is not yet a routine procedure. Their relative turbidities may be determined by means of the Zeiss nephelometer in conjunction with the Pulfrich photometer. The initial general experimental procedure follows:

The turbid suspensions of the set of glass standards to be studied (10 ml.) were placed in special blown-glass beakers or in special plane cells supplied for use with the nephelometer and that were stoppered to prevent evaporation. These test vessels were arranged in their holders in such a way that irregularities or bubbles in the walls of the vessels were not in the field of view, that is, did not intercept the beams of either the illuminating or scattered light. Special precautions were taken to stir the suspensions immediately before the nephelometric readings were made. This was done by drawing up the suspensions in 10-ml. pipettes and allowing the material to flow back so that layers that may have settled out while the temperature was being regulated were thoroughly mixed. In order to compensate for the heat radiated by the illuminating lamp and to maintain a constant temperature, cold tap water was passed through the temperature control chamber beneath the water chamber. Twice-distilled water was used to wash the chamber and to fill it to such a level that the illuminating beam of a square cross section passed entirely through the turbid suspension and the water bath that surrounds it. The incident beam of parallel light should always enter the containers in such a way that the light scattered by the turbid medium at an angle of 45° to the direction of illumination enters one aperture of the photometer for measurement.

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[1] There are, of course, other methods of approaching such problems. The material might be ashed and placed in a cored Acheson graphite electrode and an electric arc method used to produce emission spectra. Only experience will determine the method best suited to the study of any particular substance.

The stratum thickness of the 26-mm. beakers was too great for measurement with the more turbid glass suspensions, which were therefore measured in special plane cells of 2.5-mm. stratum thickness. The values of the relative turbidities of the glass suspensions in these test vessels versus concentrations are recorded in curves A and B, respectively, of figure 21. Measurements were made with light transmitted by a green L_2 filter.

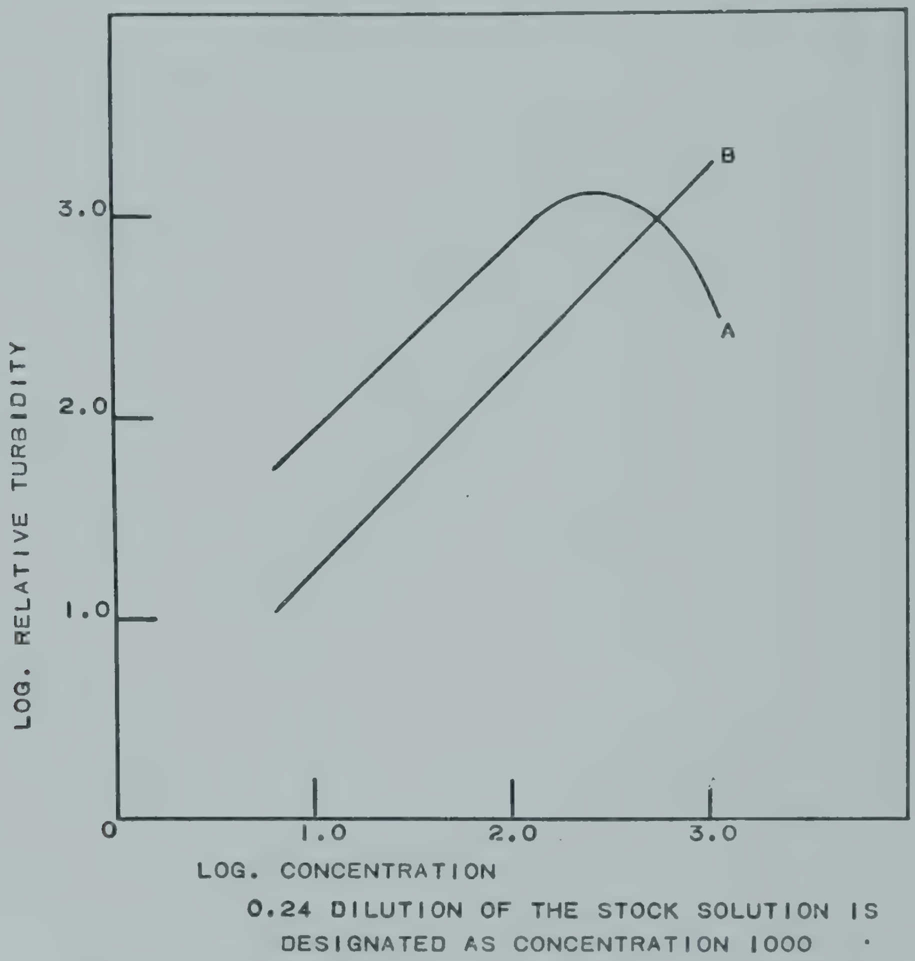

FIG. 21. RELATION BETWEEN RELATIVE TURBIDITY AND CONCENTRATION
(0.24 dilution of the stock solution is designated as concentration 1000)
A. Glass suspensions in 26-mm. beakers.
B. Glass suspensions in special plane cell of 2.5 mm. stratum thickness.
Parallel illumination and green filter L_2 used

Curve A represents the relation between the concentrations of the glass suspensions and the relative turbidities; both are expressed logarithmically. The intensity of the light scattered by the suspensions is proportional to the concentration from the lowest value to the concentration represented on the chart as log concentration 2.0000. But from this point, as the concentration increases the rate of increase in intensity becomes less until a critical concentration is reached, when it decreases rapidly. The critical concentration for the suspensions measured in the 26 mm. beaker is represented in the curve as approximately log concentration 2.4000. Near and beyond the critical concentration, the intensity of the scattered light falls off because the stratum thickness is great enough to reduce appreciably the intensity of both the illuminating beam and the scattered radiation.

Curve B represents, in a manner similar to curve A, the relationship of the logarithms of the concentrations and of the relative turbidities of the more concentrated glass suspensions as measured in 2.5-mm. stratum thickness. There is a direct proportionality between the turbidity and concentration of the suspensions and from this curve it is possible to read the values of relative turbidity for considerably higher concentrations than was possible in curve A.

When the range of concentration is considerable, it is usually necessary to resort to the use of two or more types of test vessels as described above since each type of vessel is suitable only for a particular range of turbidity. For example, very faint turbidities cannot be measured accurately with a thin stratum thickness (2.5 mm.) such as was used in determining curve B because the intensity of the scattered light is too low. On the other hand, the high degree of turbidity of some of the suspensions, if measured in vessels of 26-mm. stratum thickness, makes it necessary to take readings from the curve on the wrong side of the critical concentration. Hence, use was made of the two test vessels in obtaining data for curves A and B in order to find a range for each container within which the scattered light increased in direct proportion with the concentration—within certain limits, with the number of particles in the investigated suspensions.

The turbidity values recorded in the figure are the averages of five sets of readings made by two observers for the concentrated suspensions and of four sets of readings for the more dilute suspensions. In obtaining the data, it was necessary to resort to the use of three of the opal glass comparison intensity standards, Nos. 2, 3, and 4, which are inserted in the rotating disc of the nephelometric attachment of the Pulfrich photometer. These, in turn were calibrated with the milk glass standard block, the turbidity (0.0111) of which is known. It will be necessary to repeat this calibration of the opal glass comparison intensity standards with the milk glass standard block when turbidities of other lots of glass standards are measured, since the opal glass comparison standards may not be sufficiently constant whereas the standard milk glass block is practically invariable. Reference has been made to Zeiss instructions for some of the experimental details in carrying out these measurements (21).

SPECTROSCOPIC EQUIPMENT

Spectroscopic Instruments and Accessories

Hilger E316 all-metal quartz spectrograph with Hilger rotating sector photometer and a Spekker photometer (for ultraviolet region of the spectrum)
Hilger-Nutting spectrophotometer (for visible region of the spectrum)
Hilger large-model infrared spectrometer with rock-salt prism, Hilger thermopile, and Coblentz thermopile (for infrared region of the spectrum)
Zeiss microspectroscope (for study of minute quantities of substance and location of faint absorption bands)
Leeds and Northrup high-sensitivity galvanometer
A modified Julius suspension for the galvanometer

Measuring Instruments and Accessories

Zeiss photoelectric recording photometer (for evaluation of ultraviolet, visible, and near infrared emission, absorption, fluorescence, and Raman spectrograms)
Hilger measuring micrometer, 150-mm. travel, reading to 0.1 micron (for measuring spectrograms and microphotometric traces of spectrograms)

Measuring eyepiece with an accurate scale reading to 0.1 mm. over a range of 2 cm.
A Leitz projection apparatus available for aid in evaluation of spectrograms

Light Sources and Accessories

For continuous spectra:
- A hydrogen-discharge tube (Bay and Steiner type, glass with quartz endplates) and a 5 K.V.A. transformer to operate it
- A Hanovia fused all-quartz hydrogen-discharge tube (ultraviolet radiation). A rocking mirror will eventually be acquired for use with these tubes for quantitative work with the Spekker photometer
- A tungsten-steel condensed spark with a ¼ kilowatt transformer (ultraviolet discontinuous spectra)
- Cenco supervac pump; McLeod gauge; glass chain for use in refilling the Bay and Steiner hydrogen-discharge tube
- Nernst burner as a source of infrared radiation
- Pointolite lamp (visible radiations)
- Foucault heliostat
- 1000-watt, water-cooled, mercury-vapor lamp (General Electric Co.)
- Hanovia Analytic Quartz Lamp (for fluorescence investigation)
- Sodium-vapor lamp
- Hitchen sparking tube for solutions (with silver and graphite electrodes)
- Cooper-Hewitt mercury vapor Lab-Arc (nearly monochromatic radiations with certain filters)
- General Electric B-H_4 mercury lamp
- Carbon arc (Zeiss)
- Neon and argon glow lamps
- Geissler discharge tubes containing cadmium, thallium, sodium, nitrogen, mercury, and helium
- "Raies ultimes" powder
- Filters (mounted in metal holders):
- Eastman Kodak Co.:
  - Neutral filters
  - Spectroscopic filters
  - Special Wratten green line filter No. 77
  - Tricolor filters (red, blue, and green)
- Schott (Jena, Germany):
  - Schott B G 12
  - Schott U G 1
- Corning (Corning Glass Works):
  - Violet No. 5113
  - Violet ultra No. 5860
  - Didymium No. 5120
  - Noviol shade A No. 3389
  - Noviol shade C No. 3385
  - Noviol shade O No. 3060
  - Dark shade blue green No. 4303
  - Light shade blue green No. 428
  - Red purple corex No. 986
  - Red ultra No. 584
  - H.R. Red purple ultra No. 587
  - H.R. Nultra No. 738
  - Sextant green No. 401
  - H.R. Yellow shade yellow No. 3486
  - H.R. Red shade yellow No. 3480
  - Fluorescent Canary No. 375
- Some combinations to give "Woods light"
  - Schott U G 1 + No. 428 or Schott B G 12
  - Red ultra No. 584 + No. 428 or Schott B G 12
  - Red-purple ultra No. 587 + No. 428 or Schott B G 12
- Filters of wire gauze

Safety cage to enclose high-tension lines from transformer
Hemsalech self-induction coil (for removal of air lines in spark spectra of metals)
Miscellaneous accessories: Wollaston prism, Wallace replica grating (Michelson), Vitreosil lens and cell for fluorescence, Fused Quartz cells, 5.0 mm. (Aminco), Fused-silica absorption cells, 100, 20, 10, and 5 mm. (Hilger), Fused glass cells, Micrometer cell; Charts: Iron, copper, neon, helium, and "raies ultimes" spectra; Rock salt crystals

The following additional equipment and technics are in use or in the process of development:

Zeiss Pulfrich photometer and nephelometer and accessories for the study of fluorescence
Rotation of the plane of polarization, Schmidt and Haensch Polarimeter and Polaroid disks
Fluorescence
Emission spectroscopy
Kofler Hot Stage for determining corrected micro-melting points of substances studied spectroscopically
The Raman effect
Spectroscopic studies of solutions at low temperatures—liquid air, liquid nitrogen, and mixtures of dry ice with Cellosolve and other solvents
Chromatographic analysis (Tswett's adsorption method) and ultrachromatography
Light scattered by solutions
New light sources: sodium, helium, and mercury-vapor lamps
Photoelectric cells
Automatic recording of infrared spectra

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CHAPTER 12

ESTIMATION OF POTENCY BY BIOLOGIC ASSAY

The term *biologic assay* embraces all forms of tests of potency of physical or chemical agents acting alone or in combination in biologic systems. An extensive recent review has been presented by Bliss and Cattell (1), who define the subject broadly enough to include biologic reactions not involving intact cells, such as serologic tests *in vitro*. Some of the fundamental concepts are treated in an article by Irwin (2-a) and the discussion (2-b) following; especially interesting is Neyman's contribution (2-c). A chapter entitled "The Measurement of Immunity Reactions in the Living Animal" is introduced by Topley and Wilson (3) by the statement, "It is impossible to understand the present position of immunology, with its mixture of established fact, half-knowledge, hopeful guessings and frank bewilderment, without an adequate grasp of the difficulties involved in measuring immunity reactions in the living animal, and in assessing the significance of such measurements when they have been obtained." As Bliss and Cattell point out, the field has been developed through collaboration of statisticians and experimental biologists.

Even under apparently standardized test conditions, biologic reactions in some individuals may be of such an unexpected character or so exaggerated that it becomes questionable whether they are relevant or not. Judging response may be too subjective to make decision about individual responses practicable. The limitations in our knowledge of biologic reactions may imperil judgment in selecting individual features of a test plan. On the other hand, an improper selection of statistical technics may introduce unnecessary difficulties or, even worse, yield misleading results. The subject has therefore seemed of sufficient importance to justify the inclusion of recommenced methods in the present edition.

Certain general topics and inter-relations are developed first below, followed by specific consideration of the evaluation of *quantal* assays (where the response is all or none) and other assays which may be designated as *gradational*. End-point calculation procedures are illustrated in the specific discussions. Experimental planning, sampling, and some probability relations are also discussed, partly in fine type to facilitate a rapid review of the salient features.

In tests upon animals with chemical agents, including antigens and antibodies, it usually appears best to vary the dose (D) of any given reagent in geometric progression, the successive increments in log D being equal, and in any given test to administer in each case the prescribed dose D to an equal number (n) of animals. Each *case* here corresponds to an ultimate class or category in which n animals (subjects) are all to be given the same treatment in so far as may be practicable and observed for certain given kinds or degrees of response according to the test plan. Suppose there are H ultimate categories possibly testing various reagent preparations in a given test-plan and n subjects are required in each case; then $n \cdot H$ animals are required in total.

A suitable choice of species, and of the conditions of breeding, maintenance, and preparation for tests are of prime importance, as well as restrictions that may be made with regard to certain attributes of test animals such as age, weight, apparent condition, and sex (cf. Bliss and Cattell (1-a)). A pool of at least nH animals is selected according to the specifications adopted. From these, n are taken in an unbiased manner and placed in each of H cages, numbered $1, \cdots, H$, to correspond with the H cases under test. Two systems of selection are given below.

Unrestricted Random Sampling (4-a).—$n \cdot H$ tags (cards, capsules, or balls, etc.) otherwise alike are numbered n each $1, \cdots, H$. These are placed in a bowl, shuffled and withdrawn one at a time at random without replacement as animals are taken individually from the pool and placed in the correspondingly numbered cage in each instance. The term *random sampling* usually implies unrestricted random sampling.

Stratified Random Sampling (4-a).—Instead of the preceding technic, if strong reasons appear in favor of the alternative, only H tags, one to each number, may be used. All H tags are replaced after all are withdrawn and the process is repeated, round by round, until each cage has n subjects. This is not entirely a random sampling process. It yields a stratified but unbiased sample, and may be called briefly a *simply stratified* sample. Many other systems of stratified sampling are possible; for example, take the H animals of each successive *round* successively increasing in weight or other attribute, or each H animals of the same sex, etc., in any prescribed manner.

Difficulties in probability calculations, sometimes apparently insurmountable, are introduced if a *random* sample is not employed, but a stratified sample might be somewhat preferable in certain situations. Thus in dealing with a pool of animals, some of which have greater ability to elude the sampler so that other less agile subjects are first picked for distribution in the cages, the stratified sample assures having one of the first H in each cage and likewise one of each successive set of H animals so taken. Such agility, if related to variation in resistance one way or another, or suspected of some relation, might throw the weight of judgment in favor of a stratified sampling technic.

In some situations only approximations to such systems of sampling are practicable, as in certain experiments using aseptic drosophila larvae as subjects for estimation of radiation effectiveness (5–7). Wells in paraffin blocks were used instead of cages as above, and the larvae were distributed with the aid of a small brush a few at a time to each well in turn, until at least twenty rounds had been made. In each case about 400–600 subjects were used as judged by rough estimates of approximate mean number of larvae transferred at a time multiplied by the number of rounds.

Types of Response.—The criteria used in certain assays as a basis for judging reactions of animals to given doses of reagent under test may consist of *all or none* responses in each animal such as death within a given interval or survival. These are called *quantal assays.* Otherwise, an assay may utilize as criterion the *reaction time* (T) required for a given response (1-b, 8, 9); or the amount (A) of a *graded response* (1-c) may be used where the extent of reaction is measurable and is either an increasing or a decreasing function of the dose D administered, at least within a prescribed range (7). These may be called *gradational assays*; in each individual the reaction time or extent is used in relation to the dose of reagent as a concomitant variable (*variate*). Another type of gradational assay is indicated in the course of the following paragraph.

Individual Effective Dose (2-d).—In certain types of assay it may be supposed that for each animal there exists under conditions otherwise fixed a minimum dose of reagent which would produce the prescribed critical response (for example, the smallest dose necessary to produce death of a given *individual* within a prescribed time interval after administration). Assumption of the existence of a minimum effective dose characteristic of a species has led to a great deal of confusion in attempted biologic assay (2-d). However, the concept of *individual* minimum effective dose is useful, although it is rare that a method has been devised for its precise determination. A striking example is the assay of digitalis by slow injection in cats producing a cumulative effect (10, 11). This is another type of *gradational assay*, the dose D being administered slowly, and the assumption being made that the effect is due essentially to the accumulated dose in the subject. Usually, this is not practicable or the assumption is unjustified, and, instead, n animals are all given the same dose D as indicated previously. If r of these respond critically whereas the others $(n - r)$ do not, then the minimum effective dose is D or less for the r that responded critically; and the others may be considered to have minimum effective doses greater than D unless a dose such as D could produce less effect than a smaller dose.

That this danger is real is illustrated by tests of an antiserum in various doses D in animals, all of which receive a standard injection of pneumococcus culture also. The critical response is survival for four days. Protection is provided with certain doses D against the practically certain lethal dose of the pneumococcus culture. It is possible, however, by successively increasing doses of certain antisera to produce failure to survive. This may be regarded as due to the increasing influence of another kind of activity which produces the prozone effect.

The Sampled Universe (Population).—However large the number n of animals used in each case it is usually regarded as only a sample from a larger universe of animals, called simply the *population*, that could have been selected in the same manner for the purpose. This larger universe is usually regarded as if unlimited in number.

Use of Various Types of Average.—The results on individual subjects of the sample of n animals are used to calculate a value called an *average* to represent the sample. Various forms of average may be used (12) such as the mean (6, 11), the harmonic mean or its reciprocal (8), the geometric mean, or the median (7, 13, 18). Such an average is important as a rule only in as much as it may be used as an approximation of the corresponding average that supposedly might be obtained for the *population* (sampled universe).

The Notion of Confidence Range.—It is highly desirable to be able to make some statement about the probability that the population value would be found to lie in a given interval. Such intervals are called confidence ranges, but often the probability cannot be stated precisely but only a number called the *coefficient of confidence* which is somewhat less satisfactory. In the case of the median an ideal situation exists if the sample may be regarded as a random sample of either a finite or an infinite population; and it has been shown how the desired probability, called *specific confidence*, may be evaluated (13). Other uses of the notion of confidence (fiducial) interval are discussed by Fisher (14, 15), Neyman (4-b, 16), and Clopper and Pearson (17).

Median Values and Confidence Ranges.—Given a sample of n individuals from a given population, suppose the corresponding set of values (X_k) of a given variate is obtained, where $k = 1, \cdots, n$. To simplify discussion suppose no two of these values of X can be *exactly* the same. Actually, values exactly alike may be encountered, but the results obtained by excluding such a possibility from the discussion may be taken as merely approximate to the extent that like results might be recorded by a process of *rounding off* values really slightly different. For convenience, suppose the enumeration of (X_k) to be in an ascending order of magnitude $X_1 < X_2 < \cdots < X_n$. Then the median (m) of the sample values is defined as the middle value $(X_{\frac{n+1}{2}})$ if n is odd, or half the sum of the two middle values $(X_{\frac{n}{2}}$ and $X_{\frac{n+2}{2}})$ if n is even. Thus, if the middle value (or values) are known, nothing more is needed to evaluate m. In the case of reaction time observations (1-d, 7, 18), advantage may be taken of this fact to avoid prolonging observations after half the animals have responded critically to yield a value of T; there is *no error* involved in this case as in taking

T as infinite for animals that do not respond in the period of observation and in some situations results may be found more stable in terms of the median than when the mean is used, as extreme or even erroneous values have relatively little influence on that average.

TABLE 5

Confidence ranges for the median (13)

Table of $I_{0.5}(n - k + 1, k) = \bar{P}(M < X_k) = \bar{P}(M > X_{n-k+1})$, where M is the *true median* of an infinite universe from which a random sample of n observations, (X_k) for $k = 1, \cdots, n$, has been obtained and the enumeration (k) made after arrangement of the observations in ascending order of magnitude.

	1	2	3	4	5	6	7	8	9	10 ← n	k ↓
	.5000	.2500	.1250	.0625	.0312	.0156	.0078	.0039	.0020	.0010	1
		.7500	.5000	.3125	.1875	.1094	.0625	.0352	.0195	.0107	2
20	.9999990		.8750	.6875	.5000	.3438	.2266	.1445	.0898	.0547	3
19	.999980	.9999981		.9375	.8125	.6562	.5000	.3633	.2539	.1719	4
18	.99980	.999962	.9999962		.9688	.8906	.7734	.6367	.5000	.3770	5
17	.9987	.99964	.999928	.9999924		.9844	.9375	.8555	.7461	.6230	6
16	.9941	.9978	.99934	.99986	.999985		.9922	.9648	.9102	.8281	7
15	.9793	.9904	.9962	.9988	.99974	.999969		.9961	.9805	.9453	8
14	.9423	.9682	.9846	.9936	.9979	.99951	.999939		.9980	.9803	9
13	.8684	.9165	.9519	.9755	.9894	.9963	.99908	.99988		.9990	10
12	.7483	.8204	.8811	.9283	.9616	.9824	.9935	.9983	.99976		
11	.5881	.6762	.7597	.8338	.8949	.9408	.9713	.9888	.9968	.99951	
10	.4119	.5000	.5927	.6855	.7728	.8491	.9102	.9539	.9807	.9941	
9	.2517	.3238	.4073	.5000	.5982	.6964	.7880	.8666	.9270	.9673	
8	.1316	.1796	.2403	.3145	.4018	.5000	.6047	.7095	.8062	.8867	
7	.0577	.0835	.1189	.1662	.2272	.3036	.3953	.5000	.6128	.7256	
6	.0207	.0318	.0481	.0717	.1051	.1509	.2120	.2905	.3872	.5000	
5	.0059	.0096	.0154	.0245	.0384	.0592	.0898	.1334	.1938	.2744	
4	.0013	.0022	.0038	.0064	.0106	.0176	.0287	.0461	.0730	.1133	
3	.00020	.00036	.00066	.0012	.0021	.0037	.0065	.0112	.0193	.0327	
2	.000020	.000038	.000072	.00014	.00026	.00049	.00092	.0017	.0032	.0059	
1	.0000010	.0000019	.0000038	.0000076	.000015	.000031	.000061	.00012	.00024	.00049	
↑ k	n → 20	19	18	17	16	15	14	13	12	11	

Confidence Ranges for the Median (13).—The values of X corresponding to the entire population may be considered a population or universe (U) of values of X of which the foregoing set (X_k), $k = 1, \cdots, n$ is a random sample (if the original sampling was random). Suppose this to be the case, and let M be the unknown median of the population U. Then for any value in the set (X_k) which is arranged in ascending order, the probability that M is less than X_k is denoted by $\bar{P}(M < X_k)$. This has been evaluated in any case (13), and for an infinite universe U found to be equal to

$I_{0.5}(n - k + 1, k)$ in terms of the function of Pearson (19) and Müller (20) which has been tabulated (21).

The probability that M is greater than the k-th value from the other end is the same; i.e., $\bar{P}(M > X_{n-k+1}) \equiv \bar{P}(M < X_k) = I_{0.5}(n - k + 1, k)$. In table 5 are given values of $\bar{P}(M < X_k)$ for $1 \leq k \leq n \leq 20$. Obviously $\bar{P}(X_k < M < X_k') = \bar{P}(M < X_k') - \bar{P}(M < X_k)$. It should be noted that extreme values (i.e., above X_k' or below X_k) are not required for the evaluation of the confidence interval (X_k, X_k') and this corresponding confidence value. Usually X_k' is taken as the k-th value from the other end; namely, X_{n-k+1}, and $\bar{P}(X_k < M < X_{n-k+1}) = 1 - 2 \cdot \bar{P}(M < X_k)$ is the probability that M lies in the given interval.

Note: In the article cited (13), on the last page line 2 read $\dfrac{\text{“}N + a\text{”}}{2}$ instead of $\dfrac{\text{“}N - a\text{”}}{2}$, and on line 4 read $\dfrac{\text{“}N + 1\text{”}}{2}$ instead of $\dfrac{\text{“}N\text{”}}{2}$. Also in relation (13) the symbol “Z” should not appear.

QUANTAL ASSAY

Plan for One Reagent Variate (*D*).—Suppose, to simplify the discussion, that we are dealing with only one reagent, for example, toxin given to n animals in each case, the doses being respectively $D_1, D_2, \cdots, D_h$, and in a constant ratio $R \equiv D_i/D_{i-1}$ following the plan outlined above. As is required, then there is a constant increment in log dose.

$$\text{(1)} \qquad \text{Let } L_i \equiv \log D_i \text{ and } d = \log R$$

Then, the constant increment in log dose is

$$\text{(2)} \qquad d = L_i - L_{i-1}, \text{ where } i = 2, \cdots, h.$$

Response Frequency (*r*) and Relative Frequency (*p*).—In the case of the n animals given the dose D_i in the plan outlined above, let r_i be the number reacting critically. This may be called the critical *response frequency*. Let $p_i = r_i/n$, the corresponding *relative frequency*.

Illustrative Data.—The data in table 6 are those of Mrs. Joyce Wilson and Professor Topley used in two articles by Irwin and Cheeseman (22, 23). Five animals were used in each case ($n = 5$), taken at random from 350 male mice from normal stock (individual weights being 28 to 32 grams). One cage was used for each 5 mice. Seven doses of toxin were used, prepared by successive 2-fold dilutions so that a geometric progression resulted with $R = 2$. The 70 sets of 5 animals were thus used to furnish 10 replicates in each case, and were considered as belonging to 10 groups, here labeled A′, B′, C′, D′, E′, F′, G′, H′, J′, and K′. The same letters without the primes were used in the tables of Irwin and Cheeseman; primes are added here to avoid confusion of these with other symbols employed. Any one of these groups constitutes a set according to the plan given above for $h = 7$, $R = 2$, and $d = \log 2 \leqq 0.30103$. The *critical response* was death within a definite period of observation (four days). The corresponding observed values of r are given in table 6.

Interpolation from Moving Averages to Estimate Median Effective Dose (24).—Ordinarily, data corresponding to just one of the columns (A′ to K′ inclusive) would be available for estimation of (M) the median effective dose of the reagent. Particularly with small numbers of animals (n) used in each case, erratic and often ambiguous results attend the use of a simple interpolation between successive values of $p_i = r_i/n$ to estimate M, the dose corresponding to the ideal value of $p = 0.5$. *Moving averages* have been used widely by statisticians (25) as a means of ironing out some of the erraticity in such series of values as found in these columns. Thus, K successive values of r_i may be added and divided by $n \cdot K$ to estimate p to correspond to the mean of the K values of $\log D_i$. Accordingly, let

TABLE 6

*Deaths (r_i) in ten differently labeled samples of mice injected with given doses (D_i) of the same toxin preparation.** $L_i = \log D_i$

i	L_i	D_i (mg.)	A′	B′	C′	D′	E′	F′	G′	H′	J′	K′
1	$\bar{2}.7959$	0.0625	1	1	0	2	0	0	0	1	0	1
2	$\bar{1}.0969$	0.125	2	2	0	0	0	0	0	3	0	0
3	$\bar{1}.3979$	0.25	3	1	5	5	3	2	4	2	3	5
4	$\bar{1}.6990$	0.5	5	5	4	5	4	1	3	5	3	4
5	0.0000	1.0	5	4	4	5	5	5	5	5*	2	5
6	0.3010	2.0	5	5	5	5	5	5	5	5	5	5
7	0.6021	4.0	5	5	5	5	5	5	5	5	5	5

* NOTE. Data of Wilson and Topley used by Irwin and Cheeseman (22). In another article Irwin and Cheeseman give the same table except for an apparent typographic error giving 2 instead of 5 for the third entry from the bottom and right (23).

$$p' = \frac{r_a + \cdots + r_{b-1}}{n \cdot K} \text{ and } p'' = \frac{r_{a+1} + \cdots + r_b}{n \cdot K} \tag{3}$$

where $1 \leq a \leq h - K$, $b = a + K$, and K is a positive integer, which may be called the *index span* of the moving averages. Correspondingly, using L_i for $\log D_i$, then

$$\begin{aligned} L' &= \frac{L_a + \cdots + L_{b-1}}{K} = \log D', \text{ and} \\ L'' &= \frac{L_{a+1} + \cdots + L_b}{K} = \log D''. \end{aligned} \tag{4}$$

If one-half lies in the interval (p', p'') it is possible to estimate $\log D$ for $p = 0.5$ by a simple linear interpolation, as follows:

Let $\log m$ be the estimate of $\log M$, then

$$\log m = \log D' + d\frac{0.5 - p'}{p'' - p'}. \tag{5}$$

However, all this can be done with exactly the same result simply by use of the following *general formula*:

$$\log M \cong \log m = L_a + \frac{d(K-1)}{2} + d\left(\frac{\frac{nK}{2} - r_a - \cdots - r_{b-1}}{r_b - r_a}\right), \text{ or} \tag{6}$$

$$\log M \cong \log m = L_a + \frac{d(K-1)}{2} + d\left(\frac{\frac{K}{2} - p_a - \cdots - p_{b-1}}{p_b - p_a}\right).$$

The first is used unless there is an inequality in the number (n) of animals under the various cases involved due to objective rejections such as would be required if accidental death occurred in some animals; then possibly the second may be used as an approximation.

It can be shown rigorously that relation (6) yields the same result as relations (3, 4, and 5) and that it is an interpolation if and only if the factor[1] in parentheses in the last term lies in the interval from zero to unity. The latter is equivalent in use of the first form of (6) to the requirement that $r_b \neq r_a$ and the *trial function* defined as $r_a + \cdots + r_b - \frac{nK}{2}$ lie in the interval (r_a, r_b); and it is convenient to run down the column and sum spans of $K + 1$ values of r_i, noting when this sum minus *one-half* nK is equal to the first or last value of r in the summation or between them. The index of the first value is then a in the formula above. K is chosen according to experience with a given system as are n, d, and h in the plan, with care to reduce to negligibility the chance of obtaining results that are indeterminate (as may occur, of course, under any system). Once the parameters d, n, and K have been fixed, relation (6) may be simplified by substitution of their values as constants and usually further by a collection of some terms.

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[1] In terms of this same factor (f) an estimate of the standard deviation of $\log m$ may be made from a single assay (strictly applicable only in the case of *unrestricted random sampling*, but useful for comparison with actual direct estimates from repeated assays of the same preparation, or as a preliminary estimate in default of such replicate assay experience). This is given by

$$\sigma_{\log m} \cong d \cdot \sqrt{\frac{(1-f)^2 p_a q_a + p_{a+1} q_{a+1} + \cdots + p_{b-1} q_{b-1} + f^2 \cdot p_b q_b}{(n-1)(p_b - p_a)^2}}$$

where $p_i = r_i/n_i$ and $q_i = 1 - p_i$ with n_i denoting the number of animals used in the given case; $n_i = n$ according to plan. Otherwise, the corresponding term in the numerator ($p_i q_i$) should be replaced by $p_i q_i (n-1)/(n_i - 1)$.

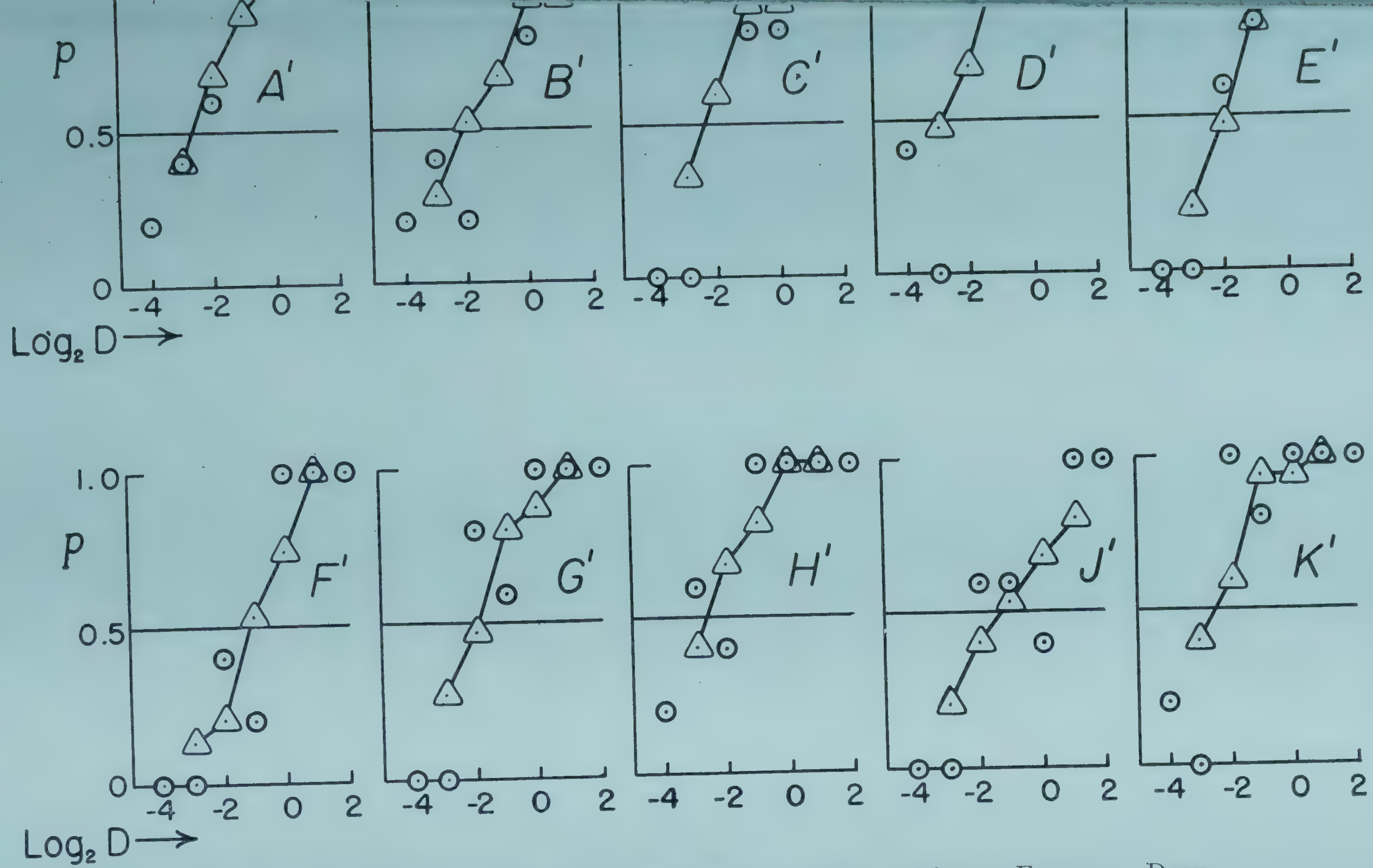

FIG. 22–a. ILLUSTRATION OF MOVING AVERAGE ESTIMATION OF MEDIAN EFFECTIVE DOSE

As the base R logarithms of $D_i(\log_R D_i)$ are integers this scale is convenient. Conversion to the base ten may be made by $\log_{10} X = d \cdot \log_R X$, where $d = \log_{10} R$. Here $d \cong 0.30103$. The circles represent values of $p_i = r_i/n$ from the data of table 6; the triangles represent the corresponding moving averages of three successive values of p_i, their join-lines form a polygon curve which crosses the 0.5 horizontal at the end-point value ($\log_2 m$). The mean of the ten values of $\log_2 m \cong -2.123$ and their standard deviation is about 0.588, corresponding to a percentage deviation in m of -33 or $+50$.

For $n = 10$, fair results have been obtained with $K = 2$ and $d = \log 2$ in protection tests with antiserum. Such a system is described and the application illustrated in another chapter (see p. 686). There relation (6) is simplified after introduction of those values of n, K, and d, and presented as (6.3). In the case of the data of table 6, where $n = 5$ and $d = \log 2 \cong 0.30103$, good results are obtained with $K = 3$. Thus, from the columns A′ to K′ successively, the following estimates were obtained (24) for $\log M \cong \bar{1}.210$, $\bar{1}.360$, $\bar{1}.285$, $\bar{1}.147$, $\bar{1}.428$, $\bar{1}.669$, $\bar{1}.428$, $\bar{1}.210$, $\bar{1}.624$, and $\bar{1}.247$. The mean of the estimates of $\log M$ is $\bar{1}.361$, and their standard deviation $s \cong 0.177$.

In figure 22a the data of table 6 are used in graphs of $p_i = r_i/n$ against $\log_2 D_i$, indicated by circles in ten diagrams corresponding to A′ to K′ of the table, each representing a complete test in itself. It is convenient to use logarithms to the base R sometimes (as in the illustration where $R = 2$). This gives values of $\log_R D_i$ successively increasing by unity. For any number X, $\log_{10} X = \log_{10} R \cdot \log_R X$ for any positive number $R \neq 1$, and $d = \log_{10} R$ by definition. Therefore $\log_{10} X = d \cdot \log_R X$ for any number X.

The triangles in the same figure represent successive values of the moving average p' for $K = 3$ in relation (3) against $\log_2 D'$ for $a = 1, 2, 3, 4, 5$; p'' is, of course, the succeeding value of p' in each instance. The triangles are joined by straight lines which cross the 0.5 horizontal at the end point value ($\log_2 m$) if $p' < 0.5 < p''$. If $p' = 0.5$ then $\log_2 D' = \log_2 m$. The graphic representation is useful only as an illustration of the process more readily performed algebraically.

Example Calculation.—As an illustration of the procedure in these calculations consider the situation in table 6. If the values of K, n, and d to be used in any system have been prescribed, then, as noted above, relation 6 may be written in a simpler form *to cover the given situation*. Here $d \cong 0.30103$, $n = 5$ and we have prescribed $K = 3$. Accordingly, as $b \equiv a + K = a + 3$, relation 6 reduces to

$$\text{(6.1)} \qquad \log m = L_a + d\left(\frac{3 - 1}{2}\right) + d\left(\frac{7.5 - r_a - r_{a+1} - r_{a+2}}{r_{a+3} - r_a}\right),$$

$$\text{(6.2)} \qquad \therefore \log m \cong L_a + 0.30103\left(1 + \frac{7.5 - r_a - r_{a+1} - r_{a+2}}{r_{a+3} - r_a}\right),$$

which apply where $K = 3$, $n = 5$, and $d = \log 2 \cong 0.30103$ as stated above. Correspondingly, the *trial function* $= r_a + r_{a+1} + r_{a+2} + r_{a+3} - 7.5$ under the circumstances and its value must lie in the interval (r_a, r_{a+3}) for an interpolation to result from application of formula (6.2).

Let attention be fixed upon column A′ in table 6 as a representation of data such as may be obtained from a given serial test. To try the suitability of $a = 1$ for an interpolation by formula (6.2) we evaluate the *trial function* as $1 + 2 + 3 + 5 - 7.5$ which equals 3.5, and as this lies in the interval from r_a to r_{a+3} (the first and last of the four successive values of r running down the column) we know that an interpolation will be the result. Next, we substitute the required values of r in the formula (6.2) which gives (as $L_a = L_1 = \bar{2}.7959$ from the table)

$$\log m \cong \bar{2}.7959 + 0.30103\left(1 + \frac{7.5 - 1 - 2 - 3}{5 - 1}\right)$$

$$\cong \bar{2}.7959 + 0.30103\left(\frac{11}{8}\right)$$

$$\cong \bar{2}.7959 + 0.4139 = \bar{1}.2098$$

which, rounded off to three decimal places, is $\bar{1}.210$ as given above.

Any greater value of a than unity would not satisfy the *trial function* test for interpolation in column A′.

Now, consider the column E′. Then, if $a = 1$ is tried, the *trial function* value is $0 + 0 + 3 + 4 - 7.5 = -0.5$, obviously not in the interval (0, 4). Trial of $a = 2$ gives the *function* value $0 + 3 + 4 + 5 - 7.5 = 4.5$, which *is* in the interval (0, 5). Trial of $a = 3$ gives the *trial function value* $3 + 4 + 5 + 5 - 7.5 = 9.5$, which is outside the interval (3, 5). Obviously, use of $a = 2$ is the only procedure to yield interpolation by application of the formula (6.2) to column E′. Accordingly, as $L_2 = \bar{1}.0969$ in table 6, the estimated logarithm of the median effective dose is here given by

$$\begin{aligned}\log m &\cong \bar{1}.0969 + 0.30103\left(1 + \frac{7.5 - 0 - 3 - 4}{5 - 0}\right)\\ &\cong \bar{1}.0969 + 0.30103\left(\frac{11}{10}\right)\\ &\cong \bar{1}.0969 + 0.3311 = \bar{1}.4280,\end{aligned}$$

which is the fifth estimate of log M given above, corresponding to column E′.

Comparison of Results by Other Statistical Methods.—Not only do the data of Wilson and Topley furnish a good basis for testing stability of estimations based on certain formulas such as relation (6), but Irwin and Cheeseman's calculations (22, 23) by other formulas furnish a basis of comparison. The probit system and maximum likelihood method given by Bliss (26) and Fisher and Yates (27) is an excellent procedure where applicable but involves indirect and comparatively difficult processes in estimation. Irwin and Cheeseman applied this method to estimation of log (L.D. 50), log M in the present notation, and obtained a mean value of $\bar{1}.361$ and standard deviation $s = 0.199$ with which the results above compare very favorably.

Kärber's method (28, 29) also was used by Irwin and Cheeseman in quest of a simple method of approximately estimating the median effective dose. However, all these authors have assumed the restricting condition that $n = r_b = r_{b+1} = r_{b+2}$, etc., and $0 = r_a = r_{a-1} = r_{a-2}$, etc., and have used an unnecessarily complicated arithmetical procedure. Exactly the same result is obtained much more simply as a special case of relation (6), when $r_b = n$ and $r_a =$ zero, *under which circumstances only* relation (6) reduces to the approximation of log M by

$$\log m = L_a + d(K - \tfrac{1}{2}) - \frac{d}{n}(r_{a+1} + \cdots + r_{b-1}). \tag{7}$$

This is equivalent to

$$\log m = L_b - \frac{d}{2} - \frac{d}{n}(r_{a+1} + \cdots + r_{b-1}). \tag{8}$$

It should be emphasized that relations (7) and (8) hold only for $r_a = 0$ and $r_b = n$.

Furthermore, it is objectionable to assume that values of r preceding the actual experience (that is, for lower doses) would all be zero or that those beyond the last would all equal n in order to provide the conditions ($r_a = 0$, $r_b = n$). It is also objectionable under some circumstances to extend K, the *index span* of moving averages, more than is necessary to provide a stabilizing effect on the estimates of log M. It can be shown that if all values of r are included already except some for lower doses all equal to zero and for higher doses all equal to n, then further extension gives the same value for log M in formula (8). However, this amounts to assuming that a temporary (four-day) immortality would be conferred upon any and all animals injected with a dose less than the least actually used. Otherwise, extension of the experiment indefinitely to use of lower and lower doses D_{a-j} for $j = 1, \cdots$ would lead by substituting $a - j$ for a in relation (8), to lower and lower estimates of log M, the limit value for M being always zero. Otherwise stated the median effective dose of any toxin would be estimated always as about zero. As it is obviously impossible to have *no toxin at all* as the effective dose, this is another objection to the system of indefinite extension of K.

This does not offer a great objection in the method of Bliss, as a weighting system is used which tends to give most of the influence to values near the median value (26, 27). Similar objection can be raised, however, to methods based on two cumulants, the sum of all values of r at or below a given dosage and the sum of all values of $n - r$ at or above this dosage (assuming that all values of r for doses below the range of the experience are zero and that above this range $r = n$ always). The ratio ϕ of the sum of these r-values to the sum of the two cumulants is used instead of p and interpolation is made as above. If the *index span* is limited by abridging the table as recommended by Reed and Muench (30) so that equal numbers of dosage values D lie above and below the calculated end point, then nearly the same smoothing effect is obtained as with the moving average, provided that both estimates are based on dosage values for an equal range of $K + 1$ indices. The process is more awkward if strictly applied, and is capable of giving absurd results if not handled with care; for example, if the successive values of r, $r_1 = r_2 = r_3 = 2$ were obtained for $n = 5$ and the summation system applied uncritically to give estimates

$$\phi_2 = \frac{2+2}{2+2+3+3} = \frac{4}{10} = \frac{12}{30}$$

$$\phi_3 = \frac{2+2+2}{2+2+2+3} = \frac{6}{9} = \frac{20}{30} \text{ then the estimate } \log M = \log D_2 + \frac{15-12}{20-12}$$

which is absurd as all actual observed values of $p = 2/5$, none are greater than 0.5.

The following is a summary of comparative results with the data of table 6, the mean estimate of log M being given, followed by the sign ± and the estimate of standard deviation for individual observations (that is for values obtained on the columns A′ to K′ individually). Irwin and Cheeseman's results (22 or 23) gave log $M \cong \bar{1}.361 \pm 0.199$ by the method of Bliss (26) and $\bar{1}.368 \pm 0.190$ by Kärber's method. Estimations by the Reed-Muench method (with 4 dosage abridgment) give log $M \cong \bar{1}.358 \pm 0.198$. The moving average system of estimation with $K = 3$ gives log $M \cong \bar{1}.361 \pm 0.177$. The moving average system rests on the firmest foundation theoretically for use in general, is the easiest to use practically and actually makes the best showing in the present experience though the margin is very slight.

GRADATIONAL ASSAY

The general plan in gradational assay involves establishment approximately of a standard curve or points for interpolation (1-b, 1-c, 2-a, 7, 8) or otherwise a definite relationship permitting approximate evaluation of potency or effectiveness of a given preparation relative to a standard (31). In establishing an average to represent the effect on a group of n animals all given essentially the same treatment, use of the median has many attractive features some already mentioned (see p. 148) and also the advantage that, if a transformation of an original variate is to be made to a univariant (increasing or decreasing) function of it, for example, to log X instead of X or $1/T$ instead of T as in the system investigated by Gard (8), then the transformation need not be made prior to estimation of a median value or confidence ranges (13), but these may be found for the original variate and transformed in the given manner to obtain the corresponding median and range-limit values for the transform variate. If no interpolation is required, as in estimating the median from an odd number of observations (n), exactly the same result is obtained. If interpolation is required (as when n is even above) then a slightly different result may be obtained according to the variate system used during interpolation. However, obvious short-cut processes exist where such differences are important, obtaining the values for interpolation by first getting the corresponding values for the original variate and transforming them in the given way, etc.

Thus in reaction-time tests the T-values may be used to obtain median T later transformed by $1/(\text{median } T) = \text{median } (1/T)$ or $\log (\text{median } T) = \text{median } (\log T)$ or otherwise as required (1-d, 18). Another advantage in the case of reaction-time tests is that the original observed values of T are usually entered in ascending order of magnitude in the record without any extra effort.

In spite of the advantages in the use of the median as average, there may be other circumstances which, in a given type of assay, indicate a preference for use of another average or of regression lines fitted to the observations. Procedures and relations in gradational assay are discussed extensively, with regard to reaction time tests by Gard (8, 9) and by Bliss and Cattell (1-b), and with regard to graded response by the latter (1-c), including a combination of graded response and reaction time, and by Irwin (2-e). Reactions where the minimum effective dose may be measured on each animal are discussed by Bliss (11) with regard to their use in digitalis assay.

DISCUSSION

The usual procedure in quantal or gradational assay is to use a standard reagent (1, 2) to furnish a relative value for the unknown, usually a standard preparation of the same active agent, such as a standard antipneumococcus serum of the same type for test of another preparation. It is rare that the biologic system on which the reagent acts may itself be used satisfactorily as a standard. A striking exception to this is furnished by the case of quantal assay employing mortality of the eggs of *Drosophila melanogaster* to X-rays which has served as a biologic indicator of dosage (32). Largely on this account this quantal assay has found more extensive service in evaluating X-ray dosage than the gradational assay system of Hussey and others (7) as well as on account of the peculiarities of the curve form, mentioned previously, which limit the range of applicability of the gradational response.

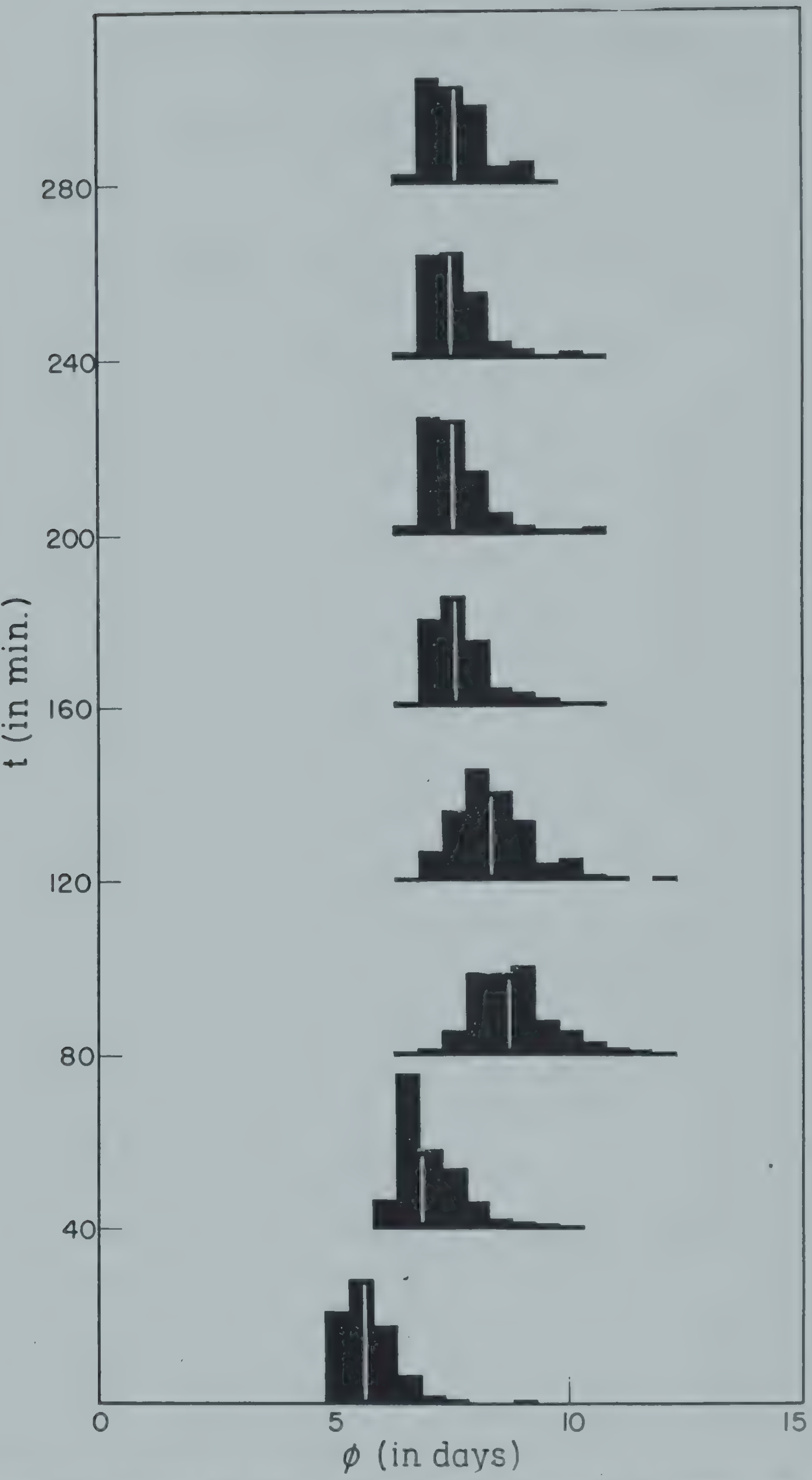

FIG. 22-b. Influence of X-rays upon prepupal period of Drosophilae. When about 3.5 days old, larvae were irradiated for the period t. Their ages at pupation (ϕ) and the median value are respectively indicated by a class-frequency diagram and a white vertical in each case, resting upon a horizontal through t. Two thirds of the distance between silhouettes corresponds to a frequency of 100 pupae formed in a half-day interval. The figure is reproduced by permission from the Journal of General Physiology, (J. Gen. Physiol., 1932, **16**, 207–220).

In any case it is well to explore a considerable range of reaction and choose a part of this where results will not be ambiguous and the sensitivity of the response is satisfactory.

A good illustration of the difficulties against which precautions must be taken in either quantal or gradational assay is provided by figure 22-b reproduced from the article of Hussey and others (7), and consists of a combination of frequency-distribution diagrams and median response curve. A *quantal* assay system could be imagined as based on responses found up to any given abscissal value (ϕ). It is easy to see how an unfortunate choice, say $\phi = 7.5$ days, would lead to ambiguities in tests and insensitivity with change in dosage above 160 minutes under the given conditions.

Illustrations of the use of sensitive systems to test the effectiveness of agents where the response is capable of precise mathematical formulation are furnished by the use of enzymes undergoing monomolecular radiochemical reaction, pepsin to test the effectiveness of β-radiation from radon after penetrating various thicknesses of an aqueous solution (33), and amylase in a similar study with ultraviolet light including an investigation of the nature of protective action of serum (34).

The complement-fixation tests (see p. 361) based on the action of complement upon red blood cells furnish an example of quantal assay where the number (n) of individuals (cells) used in any given case is very large and the resulting proportion (p) affected is estimated by comparisons of appearance of a given tube with standard tubes representing various degrees of hemolysis. The use of the probits of Bliss (or their equivalent normal deviates or of probability paper) should yield nearly the same result as does the von Krogh formula that has been employed. Furthermore, essentially the same methods (35) of construction of graphs and tables to take into account progressive changes in slope of the fitted straight lines (in the appropriate coordinate system) could be employed; thus many similar systems may furnish other situations where these methods may be used to take such progressive slope variations into account.

It is interesting to note that the *logistic function* (or *growth curve*) recommended by Berkson (36) and used also by Wilson and Worcester (37, 38) instead of the *normal deviate* or *probit* system in quantal assay is identical with the function (logarithmic form of von Krogh's formula) used in the article (35) mentioned above and other work there cited, including the original article of Wadsworth, Maltaner, and Maltaner (39). Berkson gives interesting relations applicable to fitting the curve by a method of weighted least squares. Wilson and Worcester use the method of maximum likelihood, and in another article (40) explore the more general situation where other curves or functions may be more appropriate. Further details of the work of Maltaner and associates are given in chapter 30.

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METHODS USED IN THE DEPARTMENT FOR THE PREPARATION OF MEDIA AND GLASSWARE

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EDWARD GREEN, *Senior Laboratory Worker*

PREPARATION OF MEDIA, GLASSWARE, AND DIAGNOSTIC OUTFITS. STERILIZATION

INTRODUCTION

The services of sterilization and of preparation of culture media and laboratory glassware are conveniently combined in a single department. It is convenient also to include other general services in the same department: the preparation of outfits distributed for the collection of specimens for bacteriologic, chemical, and serologic examination; maintenance of centrifuges of large capacity or special design to supplement the smaller units in the individual laboratories; and a workshop for the repair of glassware and the making of special items. In view of the fundamental character of the work, it is essential that all members of the laboratory staff be familiar with routine procedures and appreciate the potentialities of this service.

Capable members of the staff regularly assigned to the preparation of media and glassware may, in addition, be trained in the technical duties of other groups, and so be available for emergency substitution or permanent placement. A valuable aid in promoting the efficiency of the staff is an interchange in the middle of the day from the washing of glassware to the preparation of outfits, and vice versa.

When questions arise with regard to behavior of routine media, the members of the preparatory group cooperate with others interested in their study. Collaborative investigations with a view to development of new media formulas, or improvement of technic are organized and regularly conducted with supervision by a competent research assistant.

In addition to apparatus needed for small-scale operation, it has been found convenient in a large laboratory of this character to have special equipment such as a platform scale of 180-kilogram capacity; motor-driven machines for grinding and pressing meat; steam-heated water baths and steam-jacketed kettles of about 40-liter capacity for heat-treatment of culture media; a Seitz or Schriver filter-press; incubators at 35°–37°C. and 53°–55°C.; refrigerator rooms maintained at 4°–6°C. for the storage of media and at −5° to −7°C. for the freezing and storage of meat and other materials; batteries of steam sterilizers, electric ovens, and inspissators for sterilizing operations; sinks and washing machines especially designed for the cleaning of glassware; a machine for rinsing vials and small bottles; ovens for drying clean glassware; motor-driven machines for drilling and sharpening blood-letting needles, for corking tubes, for sandpapering wooden slide boxes, and for labeling mailing cases, jars and tubes, and vials containing vaccines, sera, and other preparations; an electrically heated water bath for melting paraffin; tray and platform carts for the delivery of supplies and collection of used glassware; especially designed cans for disposal of contaminated pipettes with cart for transporting cans; a glass-cutting machine; and a glass-blower's work bench equipped for general repair and construction of laboratory glassware and with facilities for working pyrex glass.

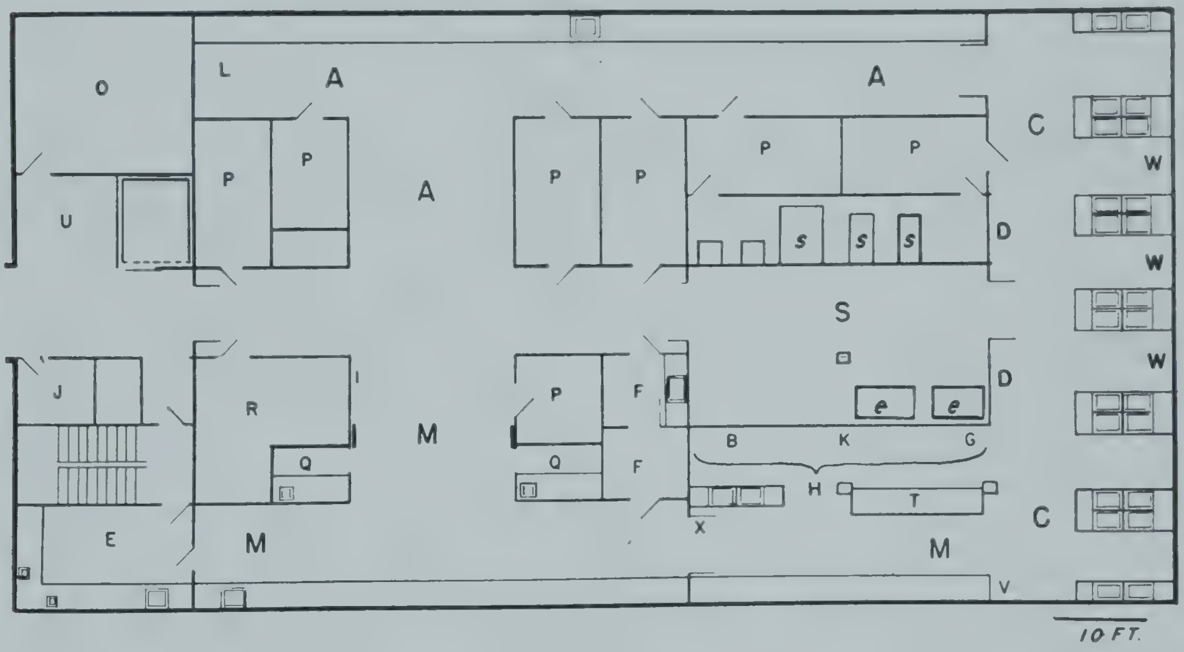

FIG. 23. MAIN FLOOR PLAN. DEPARTMENT FOR THE PREPARATION OF MEDIA, GLASSWARE, AND DIAGNOSTIC OUTFITS

O = Controlling Office
U = Distribution Center
S = Sterilization Room
(s = steam, e = electric sterilizers)
C = Glassware Cleaning Section
A = Apparatus Preparation Section
P = Storage Room

M = Media Preparation Section
H = Open Hood (over B, K, G)
F = Filtration Room
Q = Aseptic Transfer Room
R = Refrigerator
E = Experimental Laboratory
J = Janitor Service Room

L = Labeling Machine
W = Glassware Washing Machine
V = Vial Washing Machine
D = Glassware Drying Oven
G = Gas Stoves

K = Steam Kettles
B = Steam Baths
T = Filtration Bench
X = Emergency Shower
I = Incubators

The floor plan of this central service department for the preparation of glassware and media and related work is shown in figure 23. The contact or distribution office acts as intermediary between the service of preparation and the rest of the institution, particularly in routing all incoming and outgoing materials. Subsidiary to the media section are the draft-free rooms, the filtration room equipped for use of pressure or vacuum with diatomaceous earth filters, a large open hood with steam baths, kettles, and stoves beneath, sinks equipped to supply tap and distilled water, and a bench 25 inches high for funnel stands. Batteries of sterilizers are on each side of the sterilization room. The sterilization, filtration, and draft-free rooms are provided with forced ventilation. In the glassware cleaning section there are five sets of four sinks of stainless steel. Each sink is provided with a steam diffusion heater and two of the sets are also equipped with taps supplying distilled water. Large sterilizers for animal cages, a cold room maintained at −5° to −7°C., a glassware work shop, and quarters for the large centrifuges are located in the basement.

The descriptions of procedures have been designed to give enough detail to make the requirements of a given method clear to laboratory workers, without undue repetition or discussion of alternative procedures or underlying theory. Beginners should work under the guidance of experienced technicians.

CHAPTER 13

PREPARATION OF MEDIA

FORMULAS FOR MEDIA AND AUXILIARY PREPARATIONS

Suitable media for culture are sterile solutions or sols containing substances sufficient for maintenance and generation of the microorganism in question. Among the elements essential for growth are carbon, oxygen, nitrogen, and hydrogen. Salts, such as the phosphates, sulfates, and chlorides of sodium and potassium, are a further requirement, and most microorganisms need a medium of high moisture content. Some bacteria find sufficient nutriment in very simple substances, and synthetic media incorporating salts and inorganic nitrogen (nitrates, nitrites, or salts of ammonia) or amino acids as a source of nitrogen, may be employed for their cultivation. The requirements of most parasitic microorganisms are, however, relatively complex, and protein or allied substances appear essential. Originally, simple meat-infusion broths were used, but later these were supplemented by addition of peptones. Media of this type are usually solutions of noncoagulable proteins, polypeptides, amino acids, extracts, salts, and carbohydrates. For some purposes meat extracts that may be obtained from commercial sources and that are essentially condensed products of evaporation of infusions may be used instead of meat infusion. More recently, too, it has been demonstrated that some of the newer peptones and other protein hydrolyzates are capable of supplying satisfactory nutriment for many microorganisms in the absence of both meat infusion and extract. Alkali or acid may be used to adjust the medium to a pH range suitable for bacterial growth. A clarification by some process, such as boiling, followed by filtration is usually required. The medium must be sterilized and if a heat-treatment that may affect pH is used, a small portion is placed in a tube and treated simultaneously to furnish a rough check on subsequent pH of the medium. Care should be taken to avoid local overheating that may result in undesirable reactions such as decomposition or precipitation.

Some materials required in certain media would be too greatly affected by heat sterilization for the intended purposes, and, accordingly, are usually either obtained sterile or are sterilized by filtration and combined aseptically, e.g., blood, serum, ascitic fluid, blood extract, and heat-labile carbohydrate solutions. Usually in the filtration, air pressure is applied with volumes of 500 ml. or more—otherwise vacuum as described under "Filtration of Prophylactic, Therapeutic, and Other Preparations," p. 809. Sera and ascitic fluid are sterilized in this way as soon as practicable after receipt.

Amounts of material not greater than 200 ml. are filtered and delivered aseptically in one operation into a suitable bottle for storage. Small amounts are removed for sterility test, about 1 ml. each to 20-ml. amounts of aerobic and anaerobic sterility broth (*F3:A* or *B*) and semisolid meat-infusion agar (*F32:A* or *B*). Larger amounts of material sterilized by filtration are kept one day at room temperature and then tested by inoculation of 2 to 3 ml. in about 20 ml. of each of the sterility broths and in a bottle with 80 ml. of the semisolid agar. If no contaminating microorganisms are detected, the material is dispensed aseptically, with tests for sterility of material from the dispensing tip taken at approximately equal intervals during the course of the operation. The first test sample is taken in the two sterility broths as previously, after material is dispensed in the first bottle, or other container. When about one-sixth of the total volume is dispensed, a test in the semisolid agar is made, also as previously, and alternately thereafter (when about two-, three-, four-, five-sixths, and nearly all is dispensed) tests are made in the two broths and in the semisolid agar, ending with a broth test.

Materials that are not heat-sterilized are tested for sterility when they are added to other sterile materials, about 1 ml. of each remnant being inoculated into about 20-ml. amounts of each of the sterility broths and of the semisolid agar.

Media to be used for differential tests of biochemical activities of microorganisms should be prepared with care to avoid or eliminate interfering substances. For example, when the ability to ferment a specific carbohydrate is to be tested, the medium should be sensibly free from other carbohydrate impurities that might be utilized by the microorganisms instead of the test substance. This is particularly important since development of acid is commonly used to detect fermentation with the aid of an indicator. Preliminary *Bacterium coli* fermentation is frequently used to render basal preparations free of fermentable materials. Obviously, the purity of added carbohydrates should be tested.

Certain media should be tested biologically. If not indicated under the formula, such tests are described in conjunction with the methods of use. Ordinarily, culture media are used soon after preparation or else stored at 4°–6°C. Media stored for long periods should be protected against evaporation, usually with a paraffin seal, paraffined corks, cotton-cork combination plugs, or rubber stoppers. Flasks may be capped with paper, fishskin, or Cellophane to guard against dust.

GENERAL DIRECTIONS

MATERIALS

The following grades of materials that by experience have proved satisfactory are regarded as standards unless otherwise noted. Where a particular grade or brand of an ingredient has been found especially satisfactory, it is specified in the formula.

Water—distilled unless otherwise specified

Chemical substances—chemically pure (C.P.) grade if available. Where the presence of contaminating substances in the chemical may be an important consideration, products that conform to specifications of the American Chemical Society (A.C.S.) and bear a label giving the analysis should be employed.

Alcohol—indicates ethyl alcohol unless otherwise designated, and concentration in per cent indicates volume per cent; i.e., the number of milliliters of 100-per-cent ethyl alcohol per 100 ml. of solution.

Alcohol, 95 per cent—(by volume implied) denotes the standard of the U. S. Pharmacopeia (U.S.P.). It is 92.3-per-cent C_2H_5OH by weight.

Peptone—Bacto

Meat extract—Lemco or Bacto

Meat—good quality, fat and bone free, finally trimmed to remove fat and fascia as completely as possible.

Agar—a purified product, with satisfactory gelling properties when employed in concentrations of 1–2 per cent and containing a minimum of extraneous matter and salts. Each shipment of agar is tested in control lots of selected media, such as *F50B*, before being used routinely.

Carbohydrates—C.P. grade unless otherwise noted. Carbohydrates to be employed in media for differential tests of biochemical activities should be sensibly free from all interfering substances, as determined by appropriate biologic tests. Eact lot with new preparation number is tested before being used routinely.

CONVENTIONS USED IN FORMULAS

For the sake of brevity in the descriptions, certain conventions have been adopted. They apply except where otherwise specified and are presented in the order of their use.

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Formula Numbers.—"*F*" followed by the number.

Formula Ingredients.—When chemical substances are listed, the anhydrous material is intended unless the water of crystallization is indicated. Formula amounts are given for ready reference in following the procedures, and not necessarily to represent proportions in the finished medium. Horizontal lines are used to indicate certain ingredients added in proportion to resulting yields of previous procedures.

U.S.P.—Products that conform with the requirements of the United States Pharmacopeia.

C.P.—A chemically pure grade of product. Applies to all chemicals used unless otherwise specified in a given formula.

A.C.S.—Products that meet the standards of the American Chemical Society and bear a label giving the analysis. This grade of product is specified where data concerning its analysis may be an important consideration.

Biologically Tested.—Products of a suitable degree of purity as determined by an appropriate biologic test.

***X* Degrees (*X*°).**—Temperature in degrees centigrade.

Ranges.—Ranges are indicated by giving the limits, hyphenated; if one end of the range is preferable, the corresponding limit is set in italics.

Alternatively, ranges may be indicated by writing the midpoint ± half the range interval. "Heat to a temperature between 35° and 37°C.," is written "Heat to 35°–37°" or "Heat to 36° ± 1°."

Precisely.—With the greatest practicable precision of the instrument used.

Tolerance.—In media preparation the usual tolerance on weight and volume measurements is ±2 per cent of the prescribed quantity. In pH measurements (colorimetric) the usual tolerance is ±0.05, occasionally ±0.1. Even though the errors might otherwise lie within prescribed tolerances, wherever practicable all scale instruments such as pipettes and burettes should be read to an estimated nearest tenth of the smallest ruled division.

Weigh or Measure Volume.—Where directions require a volume measurement, the weight may be found instead and divided by the density. Where weight is required, the volume may be measured instead and multiplied by the density. It may suffice to estimate density from hydrometer readings or to neglect its difference from unity, but any tolerance of such errors should be based at least upon approximate knowledge of the density. If convenient, sample volumes of the material may be weighed in a stoppered bottle. Amounts given in a formula may usually be varied proportionally without other significant effect except that occasionally longer heating periods may be required with larger quantity production, since ease of heat transmission and evaporation depend on volume and shape of the bulk material and of the container. Usually it is easier to work by use of weights with large bulks in preparation.

Solution.—An aqueous solution (or sol).

Prepared Solution.—A solution of one or more ingredients in suitable concentration prepared for use in aliquots, instead of by weighing ingredients separately for each of several preparations.

g.p.l.—Grams per liter of solution.

mg. per 100 ml.—Milligrams per 100 milliliters. Equivalent to g.p.l. $\times 10^{-2}$. *Mg. per 100 ml.* is used in preference to the expression *mg. per cent.*

***Q* Per Cent Solution.**—*Q* grams of the designated material (exclusive of water of crystallization unless definitely specified otherwise) per 100 ml. of solution; equal to 10 *Q* g.p.l.

***Q* Per Cent by Weight.**—*Q* grams of the designated material (exclusive of water of crystallization as above) per 100 grams of the mixture.

Flask.—A pyrex glass Erlenmeyer flask.

Beaded Flask.—A flask containing enough glass beads to cover the bottom.

Infuse.—Thoroughly mix a quantity of finely ground meat, residue, or other material with a specified quantity of water and allow to steep overnight at 4°–6°C. unless otherwise specified.

Strain.—Pour the mixture upon a double layer of surgical gauze in a colander, squeeze the residue firmly in the gathered cloth, and combine all the strained juices.

Boil.—Heat carefully but rapidly to the boiling point and usually maintain gentle boiling for a specified time.

Boil (or Heat as Specified) to Clarify.—Heat in the required manner till the fluid is clear and coagulation complete. The coagulum should become brown if meat or blood is present. Avoid local overheating that may result in an undesirable reaction such as decomposition.

Remove Fat.—Chill liquid media and remove solidified fat from the surface. This is done at any time when it appears needed, even if not prescribed.

Make Up Weight.—Add water until the weight total of materials is the same as at the last previous weight adjustment; e.g., before the last operation, usually heating, neglecting the weight of acid or alkali solutions added in adjustments of pH. After infusion, it is supposed that enough water is boiled off in the heating so that the weight does not exceed that required.

Make Up to Total Weight.—Add water to make up to the total weight of ingredients listed in the formula, or in a part separated by horizontal ruling, except the weight of meat or residue used in infusions.

Dissolve by Autoclaving.—In the case of shredded agar and water, steep overnight at 4°–6°C. if the time is available. Weigh and then autoclave thirty minutes, or longer as needed. Check weight to detect excessive loss. Two-per-cent loss is tolerable, but more than this is attributed to boiling over, and a correction is introduced accordingly. However, with proper technic in autoclaving this should rarely be necessary.

Heat to Dissolve.—Heat, usually over a free flame or on a water bath, until the materials are dissolved.

Melt.—Melt complete or basal agars that are required for pouring or admixture by heating in steam at 100°C. for thirty minutes or longer as needed.

pH.—See "The Determination of Hydrogen-Ion Concentration," p. 101.

Determine pH.—Use Pyrex tubes of precisely the same diameter (16 mm.) hereafter designated "pH tubes." In tube A put a 5-ml. sample, 10 ml. of freshly boiled distilled water, and 0.5 ml. of the appropriate indicator solution; and in another tube, B, put 5 ml. of sample and 10 ml. of water. Put in another tube, W, about 15 ml. of water, and have available a suitable set of standard tubes, S, each containing 15 ml. of prepared buffer mixture of known pH and 0.5 ml. of the same indicator solution as used above. The standard set should cover the required pH range at intervals of 0.2. By use of diffuse light from a suitable source and a comparator block, see p. 102, a color comparison is made between light transmitted through tubes W and A in succession, and that similarly transmitted through one of the standard tubes, S, and B. The tubes and contents should be at room temperature. A match is obtained, or pH estimated to the nearest 0.05 (one-quarter, one-half, or three-quarters of the interval between successive standard tubes). If B is neither colored nor opalescent, a direct comparison between A and the standard tubes may be made instead. It is a great aid to have a suitable artificial light source.

Titrate to Required pH.—In pH tubes, set up A and B as for determination of pH as if it were at the required point. Compare with appropriate standards at or within 0.2 of the required pH, and if necessary add alkali or acid (usually 0.05 *N* NaOH or 0.05 *N* HCl from a burette) until a match or judged interpolation between standards indicates a satisfactory approximation to the required pH. The titer is expressed by t, which may be either positive or negative. Let $t = x - y$, where x ml. of 0.05 *N* NaOH is equivalent to the alkali used, and y ml. of 0.05 *N* HCl is equivalent to the acid used. The titer t is accordingly positive if alkali was required, negative if acid was required, and zero if nothing was required for adjustment. Usually either y or x is zero, and $t = x$ or $-y$ accordingly.

Adjust pH.—Titrate a 5-ml. sample to the required pH, and let t be the titer as above. Then add x ml. of *N* NaOH per 100 ml. of medium for adjustment, or y ml. of *N* HCl instead, if t is negative. Determine the resulting pH and, if necessary, repeat the process till the pH is as required. Usually it is preferable to avoid adjustments of media with acid, since local reactions with acid, such as precipitation of proteins, may occur before mixing can be effected.

Buffer Value.—A rough estimate of the buffer value (β) is usually possible from titration data obtained in pH adjustments, see p. 101.

Check Chloride Content (Concentration); Titrate with $AgNO_3$.—Put precisely 20.0 ml. of the solution into a clean evaporating dish, add 1 ml. of 5-per-cent potassium chromate as indicator, and titrate with 0.1 *N* $AgNO_3$ till a faint red tinge is obtained. Approximately,

1 ml. of 0.1 *N* $AgNO_3$ is equivalent to 0.00583 gram of NaCl;
is equivalent to 0.00555 gram of $CaCl_2$.

Always make duplicate titrations. If less than 20-ml. sample is taken, make up the difference with distilled water.

Determine the Reducing Sugar Content.—In the preparation of certain meat infusion broths, as *F3*, *F4*, *F5*, and *F5.5*, it may be advisable to make an approximate determination of the content of reducing substances in the infusion that affect a given reagent expressed as glucose equivalents; and, if necessary, to supplement these substances by the addition of an appropriate amount of glucose.

The quality of a meat infusion broth for the growth of some microorganisms and in particular for the pneumococci and streptococci is to a large extent dependent upon the content of reducing substances of the infusion. Broths prepared from infusions which have a content of reducing substances equivalent to from 0.1 to 0.15 per cent glucose are usually satisfactory whereas those from infusions with a content of reducing substances less than the equivalent of 0.075 per cent glucose yield irregular and usually scanty growth.

The lack of reducing substances in a meat infusion can be compensated, at least in a large measure, by adding glucose to the infusion and peptone before the first heat treatment. In the case of broth for the cultivation of pneumococci, the reducing sugar content in glucose equivalents usually should not exceed 0.175 to 0.2 per cent; otherwise too rapid growth and autolysis may result.

The following modification of Benedict's qualitative test (p. 514) for the presence of reducing sugar has proved useful as a rapid method for estimating the glucose equivalent of reducing substances in meat infusions and broths.

To a constant amount, 1 ml. of Benedict's qualitative reagent (p. 31) in 11- by 75-mm. test tubes add several amounts, varying from 0.02 to 0.3 ml., of the test material. Mix the contents of each tube thoroughly and cork loosely. Heat in a boiling water bath for exactly five minutes, then cool in a cold water bath for five minutes before reading. By testing a series of standard solutions which contain 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 per cent glucose in 1-per-cent peptone, an approximate relation can be established between the glucose content of a given solution and the least amount of it which will produce a distinct change in the color of the reagent from blue to a bluish green or green with the gradual formation of a small amount of yellowish or reddish precipitate. Each observer, however, must establish this relation to his own satisfaction.

With infusions and broths a preliminary test is first made with 0.05, 0.075, 0.1, 0.2, and 0.3 ml. of the test material to determine whether the glucose equivalent of reducing substance is less than 0.05 per cent or 0.15 per cent or more. If the results indicate a glucose equivalent of from 0.1 to 0.15 per cent or more, a second test is made with smaller quantities of the test material, as 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 ml. and with corresponding quantities of a standard 0.1-per-cent glucose solution containing peptone.

The glucose equivalent may then be estimated as follows:

$$C = \frac{v(0.1)}{V} \text{ per cent}$$

where C is the concentration of glucose equivalent to the reducing substances in the test material, v is the volume (in ml.) of the standard 0.1-per-cent glucose solution used to produce a convenient slight but distinct color change with 1 ml. of Benedict's reagent and V is the volume (in ml.) of the test material required to produce an approximately corresponding reaction.

The approximate reactions that may occur are summarized in table 7.

Determine Iron Concentration.—In the preparation of certain media such as *F13.1* and *F20*, where the presence of too much iron has been found to be detrimental, it may be advisable to test the medium or solutions of ingredients such as carbohydrates which are to be incorporated later, to detect the presence of more than permissible amounts of this element. In the following procedure the greater part of the iron present is removed by adsorption on a precipitate of calcium phosphate, the precipitate is then dissolved and the iron determined colorimetrically. (See *F13.1* (11).)

Transfer precisely 20 ml. of the filtered test material at pH 7.8–8.0 to each of two 50-ml. centrifuge tubes. If the test material is a culture medium, add 0.2 ml. of a 10-per-cent $CaCl_2$ solution (*F61*); a simple aqueous solution of a carbohydrate, add 0.2 ml. $CaCl_2$ and 0.4 ml. of a 10-per-cent solution of K_2HPO_4. Mix well and heat in a boiling water bath for three or four minutes. Centrifuge, discard the supernatants, dissolve each precipitate in 1 ml. of approximately 25-per-cent acetic acid (25 ml. glacial acetic acid +75 ml. distilled water), and transfer to 15- by 100-mm. test tubes of precisely the same diameter and tint and graduated at 3 ml. Wash each centrifuge tube with two successive 0.5-ml. amounts of the acid and add to the tube containing the corresponding precipitate. To one tube, A, add 0.4 ml. of a 0.1-per-cent solution of dipyridyl in 10-per cent acetic acid, and a trace (approximately 2 mg.) of glycin photographic developer (parahydroxyphenylamino acetic acid), then make up to 3 ml. with distilled water. To the second tube, B, that serves as a color control on the solution of the precipitate, add distilled water to bring the volume to 3 ml. Allow the tubes to stand for ten minutes, then compare the color in tube A with that of the standard tubes, S, using a comparator block as in the method for determination of pH. If any insoluble matter remains suspended in tubes A and B, centrifuge the tubes before making the comparison with the standards.

Filter through Paper.—Filter through usually a double layer of a good grade of pleated white filter paper with a soft creped surface. Where it is not objectionable, a small wad of absorbent cotton may be placed in the funnel as a support. Where a clear filtrate is required, refilter through the same paper if necessary.

TABLE 7

Equivalent concentration of glucose in per cent

AMOUNT OF INFUSION OR BROTH	0.15 OR MORE	0.1–0.15	APPROXIMATELY 0.05	LESS THAN 0.05
ml.				
0.05	Distinct color change to bluish green or green with small amount of yellowish or reddish yellow precipitate forming slowly	May become turbid but no distinct color change occurs	Clear or only slightly turbid but no distinct color change	Clear or only slightly turbid but no distinct color change
0.075		Distinct color change occurring slowly. Small amount of precipitate		
0.1	Rapidly turns green to yellowish green with formation of a considerable amount of precipitate			
0.2		More rapid color change with formation of increasing amounts of precipitate	Distinct color change occurring slowly with formation of small amount of precipitate	No distinct color change. Some formation of whitish or greyish precipitate may occur
0.3				

AMOUNT OF INFUSION OR BROTH	0.15–0.25	0.1–0.15
ml.		
0.02	Clear or slightly turbid but no distinct color change	Clear or slightly turbid but no distinct color change
0.04	Distinct color change with formation of precipitate, the degree of reaction increasing with concentration of reducing substance	
0.06		Very slight to distinct color change
0.08		
0.10		Distinct color change with formation of small amount of precipitate
0.12		

Filter through Hard Paper.—Filter through a single layer of a hard-surfaced paper such as Whatman No. 50.

Filter through Glass Wool.—Filter through a wad of alkali-free glass wool (Corning "Fiberglas" from pyrex glass No. 719) in a funnel.

Filter through Cotton.—Filter through two layers of nonabsorbent cotton with fibers crossed in a funnel or supported on a resistant metal sieve.

Filter through cotton and gauze.—Filter through a pad made of two layers of nonabsorbent cotton laid with fibers crossed between two pieces of gauze and supported on a resistant metal sieve. Filtration of agar by this procedure usually yields a clear agar with little loss of volume. Occasionally, however, for special media, filtration by aspiration may be desirable.

TABLE 8

Determination of iron concentration

COLOR STANDARDS*			ESTIMATED IRON IN TEST MATERIAL	
Tube No.	0.0025% $FeSO_4 . 7H_2O$	Distilled water	Fe in 20-ml. sample	Fe per liter of medium
	ml.	*ml.*	*mg.*	*mg.*
1	0.0	0.6	0.0000	0.000
2	0.1	0.5	0.0005	0.025
3	0.2	0.4	0.0010	0.050
4	0.3	0.3	0.0015	0.075
5	0.4	0.2	0.0020	0.10
6	0.6	0.0	0.0030	0.15

* Use graduated 15- by 100-mm. test tubes the same as for the test material. To 2 ml. of the approximately 25-per-cent acetic acid in each tube add the respective amounts of $FeSO_4$ solution and water given above and 0.4 ml. of the dipyridyl solution and a trace of glycin. Mix the contents and let stand thirty minutes before using. These standards may be used for several weeks if tubes are kept well stoppered and stored at 4°–6°C.

Filter by Aspiration.—When filtration of agar preparations by aspiration is required, the following technic is employed:

The mouth of a wide-mouthed bottle (usually 960 ml.) is covered with three layers of nonabsorbent cotton laid with the fibers crossed between two layers of gauze, and tied firmly about the neck with twine. All straggling ends of twine, cotton, or gauze are trimmed away. One or more bottles, so prepared, are set mouth down in a suitable pot containing the agar previously melted and cooled to 50°–55°C. (If required, egg albumen may be admixed with the cooled agar, 1 gram of egg albumen dissolved in 15–30 grams of water (or 1 egg white) per kilogram of medium.) The pot and contents are placed in steam at atmospheric pressure for a time, usually sixty to ninety minutes, sufficient to drive out most of the air from the inverted bottles, and then removed and allowed to cool. Condensation of steam in the bottles aspirates the liquefied medium through the filters into them. The bottles are set upright and the filters removed to recover the filtrate.

Filter through a Filter Candle.—Filter through a candle of diatomaceous earth using the technics described in "Filtration of Prophylactic, Therapeutic, and Other Preparations," p. 806, but without special care to maintain asepsis. The alternative procedure of inverting the candle in the container of material to be filtered is particularly applicable for use with large volumes of materials. The object of the treatment is removal of living or dead bacterial debris that might later introduce a confusing element in microscopic examinations or alter the antigenic characteristics of cultural material. Media that are to be sterilized fractionally may be filtered first in this way to remove highly resistant spore forms.

Filtration through a Filter-Press.—Instead of the foregoing filtration procedures a filter-press (e.g., Seitz or Shriver) may be used where convenient with an equivalent filtration medium. This is particularly useful with large volumes.

Dispense.—Where convenient, deliver through a pinchcock-controlled rubber tube leading to a glass delivery tip. If required to dispense aseptically, operate in a draft-free room with the tip of the delivery tube shielded from falling particles. In the case of agar plates, melt the basal medium, make any prescribed final combinations, and dispense 15–20 ml. per plate.

Dispense in (Small or Large) Double-Tube Fermentation Sets.—

Small set: A tube 11 by 75 mm. inside a tube 19 by 150 mm.

Large set: A tube 11 by 75 mm. inside a tube 22 by 165 mm.

With the designated set, dispense the medium in the larger tube, insert the smaller tube inverted, and plug the larger tube with cotton. Where a precise amount of medium is not specified, use an amount sufficient to fill the inverted tube and to rise slightly less than half as high in the outer tube. After autoclaving, the inner tube should be inspected to see that it has become filled with the liquid medium.

Autoclave (Sterilize by Steam under Pressure).—Usually at 121°C. (2 atmospheres of steam pressure, gauge reading, 15 pounds). See chapter on "Sterilization," p. 239.

Sterilize Fractionally.—Usually in streaming steam at atmospheric pressure, about 100°C., for given periods on three successive days. See chapter on "Sterilization," p. 241.

Inspissate.—Heat at temperatures below 100°C. See chapter on "Sterilization," p. 241.

Sterilization Timing Scheme According to Bulk.—If a range of periods of treatment is given, use the longest period for bulks of 2 liters or more, the mean time for 1-liter bulks, and the least time for bulks under 100 ml., unless otherwise indicated.

Sterilize by Filtration.—Filter with aseptic precautions through a candle of diatomaceous earth, or pads of paper pulp and asbestos fiber, and maintain the filtrate sterile, using specified technics as described in "Filtration of Prophylactic, Therapeutic, and Other Preparations," p. 806.

Slant.—If necessary, melt agars at 100°C. and allow the medium to solidify, usually in tubes or sputum jars with the axis inclined about 15 to 20 degrees from the horizontal. If bottles are used, allow medium to solidify with bottles lying on their sides.

Determine Melting Point of Gelatin Preparations.—Test three tubes, partly filled, by slowly heating in a beaker of water, break the gel in one by a deep thrust of a rod, place a thermometer in the second, thrust in half way, and leave the third intact. Use a control thermometer in the water and stir with the intact tube. All should melt within an interval of one degree on the thermometer in the second tube.

Attach or Join Aseptically.—Fitted parts of an apparatus to be assembled may be separately wrapped and sterilized, and then joined (attached) aseptically by carefully unwrapping each in a draft-free room, flaming the parts and promptly joining them. The joints may be tied to prevent inadvertent separation.

Sterile Siphon.—A rubber stopper is fitted with a cotton-plugged glass vent and a siphon consisting of a glass bend attached to a rubber tube, usually provided with a glass delivery tip shielded by a glass bell. The dimensions are suitable for aseptic transfer of material from a given flask or bottle. The parts that may later enter the aseptic field of the assembled apparatus are protected by cotton pads enclosed in heavy brown wrapping paper and autoclaved thirty minutes (in a protective container if not to be used immediately). Aseptic attachment of the stopper to a suitable sterile flask forms a sterile siphon. (See figure 24.)

Sterile Drain.—A sterile drain is formed by a technic similar to that used in preparing the sterile siphon, but is designed for transfer of sterile material by drainage from the inverted container. The vent is a U-shaped glass tube extending nearly to the vessel's bottom when the stopper is inserted in position, and the outer arm (plugged) is about half as long. Usually a short straight glass tube is inserted into the vessel through the stopper but not allowed to extend further inside. This tube leads via a rubber tube to a glass tip and bell as in the case of the sterile siphon. Wrapping of parts and aseptic attachment is done (as described for the sterile siphon) to form a sterile drain when the vessel is inverted. (See figure 24.)

Sterile Drain with Strainer.—Instead of the glass drainage tube through the stopper, a resistant metal tube about 1 cm. diameter is used with closed butt extending about 10 cm. into the vessel beyond the stopper, the tube having about fifty perforations throughout that part of its length. The hole size is made suitable to obtain the desired straining effect. (See figure 24.)

Sterile Drain with Plate-Filling Delivery Tip.—Instead of the bell, a glass or resistant metal tube with a beveled end is used to form a shield around the delivery tip of a sterile drain for aseptically dispensing material directly into Petri plates. The shield may be made from the stem of a funnel, cut off and flanged to admit insertion of a rubber stopper carrying the delivery tube, the stopper being wired securely to the flange. Convenient dimensions are a 75- to 100-mm. length and about 13- to 14-mm. bore for the shield, and the delivery tip about 6 mm. outside diameter extending to within 5 mm. of the end of the shield. In use the tip is inserted into the slightly opened Petri plate, pointing downward with the bevel about horizontal. (See figure 24.)

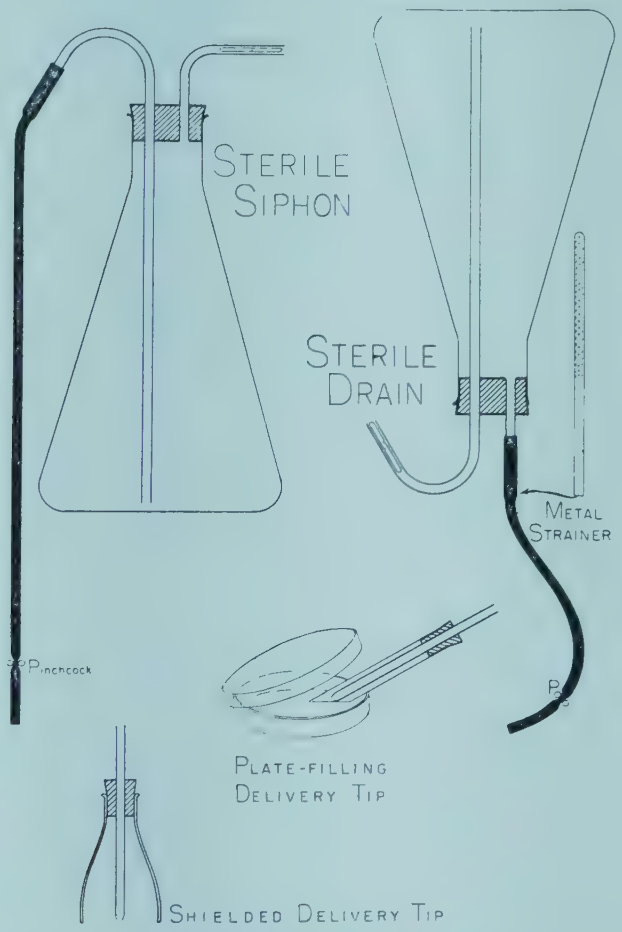

FIG. 24. STERILE SIPHON AND STERILE DRAIN (WITH SHIELDED DELIVERY TIP AND WITH PLATE-FILLING DELIVERY TIP)

Sterile Filter-Funnel.—Three folded, soft filter papers are placed in a glass or resistant metal funnel; to the tip is attached a short length of rubber tubing, the other end of which is wrapped in a wad of nonabsorbent cotton between two layers of gauze. A metal cover is secured over the funnel top by two small spring coils, the cover being provided with an inlet tube set so as to deliver incoming material to the side of the funnel. The top of this tube is wrapped as is the delivery end of the apparatus, and the whole assembly autoclaved forty minutes. (See figure 25, A.)

Filter and Redispense Aseptically.—In the flask containing the material insert a sterile siphon without the belled glass tip, and join aseptically to the inlet of a sterile filter-funnel. In turn, join this aseptically to an extra inlet tube through the stopper of a flask provided with a sterile siphon with a shielded delivery tip. Dispense the material aseptically as it becomes available. (See figure 25, A.)

Combine and Dispense Aseptically.—A rubber stopper gauged to fit the neck of the container of medium to which additions are to be made is provided with a siphon with glass-belled delivery tip, a glass vent, and an inlet tube that extends below the neck of the container but above the final level of the contents. This inlet tube in turn is connected by rubber tubing to the outlet tube of a drain, the stopper of which is gauged to fit the neck of the container of material to be added. The parts of the apparatus that may later enter the aseptic field of the assembled apparatus are wrapped and sterilized as described for a sterile siphon. The siphon is attached aseptically to the bottle or flask of medium, the drain similarly to the second container. This vessel is then inverted to allow its contents to

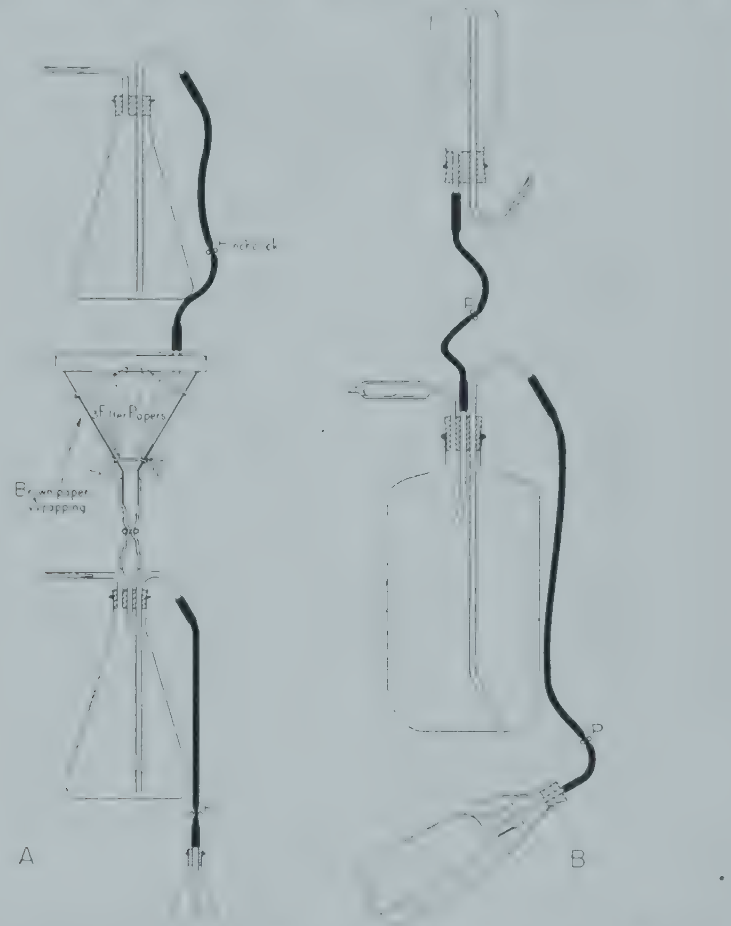

Fig. 25. A. Aseptic Filtration and Redispensing System; B. Apparatus for Combining and Dispensing Aseptically

drain into the container of medium. When this operation is complete, the outlet tube from the drain is closed by means of a pinchcock and the combined medium thoroughly mixed and dispensed aseptically. (See figure 25, B.)

Aseptically Transfer Egg Contents.—Clean the eggs thoroughly with wet gauze, place in a container, cover with 80-per-cent alcohol (by volume), and let stand for two hours. Decant the alcohol. Dry the eggs by cautious flaming and then, with aseptic precautions, carefully break the shell in half and drop the whole contents or the separated yolk as required into a sterile beaded flask.

Incubate.—Keep for the specified time in a thermoregulated bath (usually an air oven, water bath, or a refrigerator), the mean or range of the bath temperature usually being given.

Inspect.—Check grossly against contamination; if implied, against any bacterial debris or other sediment.

Store.—Usually keep in a refrigerator at 4°–6°C.

FORMULAS FOR MEDIA AND AUXILIARY PREPARATIONS

Formulas are grouped, in so far as practicable, in accordance with similarity of ingredients and methods of preparation, approximately as follows*:

I. Agar-Gelatin-Free Media
    Meat Infusions (*F1*)
    Meat-Infusion Media (*F2–F13*)
    Meat-Extract Media (*F14–F15*)
    Meat-Free Media (*F16–F29*)
II. Agar and Gelatin Media
    Gelatins (*F30–F31*)
    Meat-Infusion Agars (*F32–F47*)
    Meat-Extract Agars (*F48–F52*)
    Meat-Free Agars (*F53–F57*)
III. Other Preparations (*F58–F82*)

As far as possible, pertinent reference to the literature has been made for each of the formulas, although many formulas have been more or less adapted to suit a general plan of operation in a large laboratory.

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* With few exceptions formula numbers used in the second edition (1939) have been retained for media that are the same or essentially the same and new formulas have been given numbers with decimals so that the grouping and order remain the same as in that edition. Accordingly, the range of any group should be considered as extended to include numbers less than 0.5 beyond that given. *F13*, *F41*, *F42*, and *F57* have been omitted, since their use has been discontinued.

I. AGAR-GELATIN-FREE MEDIA

F1A. Meat infusion

Meat	450 grams
Water	1000 grams

Procedure is the same as for *F1B* except that the meat is given an additional pressing in a meat press and the residue is used in *F1E*.

F1:B, C. Meat infusion, concentrated: C. Sugar-free

Meat	900 grams
Water	1000 grams

Infuse, strain, and weigh combined juices. If less than 1 kg., make up the difference preferably with juice pressed from the residue, otherwise with water. Use the meat residue for *F1D*.

B. Boil fifteen minutes or more to clarify. Strain again, filter through paper, make up weight, and adjust pH to 7.2. Dispense and autoclave twenty to thirty minutes. Store.

C. Adjust pH to 6.8–7.0. Heat juice to 35°–37° and add 10 ml. of an 18- to 24-hour broth culture of *Bact. coli*. Incubate at 35°–37° overnight. Heat in steam at 100° for one hour or more. Strain, filter through paper, and make up weight. Fill three Smith fermentation tubes. Dispense the remainder in convenient amounts, and proceed with fractional sterilization (three 30-minute periods). Include the Smith tubes in the first day's treatment. Then cool and inoculate them with *Bact. coli*, and incubate overnight at 35°–37°. If appreciable amounts of gas are produced, reclaim the infusion immediately, pool, readjust pH to 6.8–7.0, reinoculate with *Bact. coli*, and repeat the treatments and tests as above until the infusion is sensibly sugar-free, and subsequently sterilized fractionally, three 30-minute periods. Store.

F1:D, E. Meat infusion, residue

	D	*E*
Meat residue (from *F1:B, C*, or *F11*)	900	0 grams
Meat residue (from *F1A*, *F4*, *F5A*, *F5A′*, or *F12*)	0	1300 grams
Water	1000	1000 grams

Infuse overnight at 4°–6°, then at 45°–50° for thirty minutes with occasional stirring. Boil to clarify. Strain and filter through paper. Remove fat if required. Make up weight to 1 kg. Adjust pH to 7.2. Dispense and autoclave twenty to thirty minutes. Store.

F2:A. Meat-infusion broth

Meat infusion (*F1:A*, *D*, or *E*)	1000 grams
Peptone	10 grams
Sodium chloride	5 grams

Add the peptone and salt and dissolve with stirring. Adjust pH to 7.6–7.8. Autoclave in bulk five minutes, make up weight, filter through paper, dispense as ordered, and autoclave at 115°–118° twenty to thirty minutes.

F2B. Beef-infusion broth with tellurite and ascitic fluid

Aseptically combine 300 ml. of beef-infusion broth (*F2A*) with 60 ml. of sterile ascitic fluid and 3.6 ml. of potassium tellurite solution, as used in *F40C*, and mix well. Dispense aseptically 2.5-ml. amounts in 15- by 100-mm. test tubes, incubate forty-eight hours at 35°–37°, inspect, and store.

F2C. Beef-infusion broth with serum and tissue (1) (for Treponema)

Remove guinea-pig livers with care to avoid rupture of the gall bladder, and mince into fine particles. In 16- by 133-mm. test tubes put enough of the liver to cover the bottom. To each add enough beef-infusion broth (*F2A*) to fill one-third of the tube, autoclave twenty minutes, and cool. Add aseptically enough sterile horse serum to fill the tube two-thirds. Rotate gently to mix the fluids, incubate seventy-two to ninety-six hours at 35°–37°, and inspect.

NOTE: Immediately before inoculation the medium is made semisolid by heating slowly in a water bath to 100°. Remove as soon as the desired gel is formed.

F3: A, B, C. Meat-infusion broth: A. Beef and B. Horse meat (for sterility tests); C. Special beef or horse meat (for culturing blood)

	A	*B*
Beef	450	0 grams
Horse meat	0	450 grams
Peptone (Difco proteose)	10	10 grams
Sodium chloride	5	5 grams
Glucose	1–1.75	1 gram
Water	1000	1000 grams

C. Use the ingredients throughout as listed for *A* above or else use throughout those listed for *B* above.

A, B, C. Infuse the meat overnight at 4°–6°, then at 45°–50° for one hour. Boil forty to fifty minutes to clarify.

A and *B*. Strain, *chill, and remove fat.* Filter infusion through paper and make up weight to 1 kg. (store large amounts overnight at 4°–6°). *A*. Determine the reducing sugar content of the infusion. *A* and *B*. Add the peptone and salt and dissolve with stirring. *A*. Add the required amount of glucose.

Infusions from beef usually have a lower content of reducing substances than those from horse meat. To compensate for this, glucose is added to beef infusions with the peptone before the first heat treatment depending on the reducing sugar content of the infusion. If the reducing sugar content of the infusion is equivalent to from 0.1 to 0.15 per cent glucose, add 1 gram per liter; if approximately 0.075 per cent, 1.5 gram; if less than 0.05 per cent, 1.75 gram.

A and *B*. Adjust pH to 8.0–8.2, and autoclave in bulk 20- to 40-kg. amounts forty to sixty minutes. Make up weight. *B*. Add the glucose. *A* and *B*. Filter through paper, dispense as required, usually for aerobic tests in 22-ml. amounts in 22- by 165-mm. tubes, and for anaerobic tests in 21-ml. amounts in 22- by 165-mm. tubes previously charged with an amount of paraffin-mineral oil mixture (*F79*) sufficient to provide an adequate seal after sterilization. Autoclave at 115°–118° for thirty minutes. Incubate at 35°–37° for forty-eight to ninety-six hours and inspect. Store at room temperature and inspect again just before use.

C. Filter through glass wool. Make up weight to 1 kg. Add the peptone, salt, and glucose and adjust pH to 8.0–8.2. Boil for five minutes, filter through glass wool, dispense as required, and autoclave at 115°–118° for twenty minutes.

F4. Beef-infusion broth (for pneumococci)

Beef	450 grams
Peptone	10 grams
Sodium chloride	5 grams
Water	1000 grams

Infuse the meat with nine-tenths of the water. Strain, pass the residue through meat press, and use in *F1E*. Combine juices, which should weigh 1 kg. or slightly more. Dissolve the peptone and salt in the remaining water and admix with the juices. Determine the reducing sugar content and if advisable add glucose at this point.

If the reducing sugar content of the infusion is equivalent to 0.125 per cent glucose or more, no glucose is needed; if approximately 0.1 per cent, add 0.5 gram glucose per kg.; if approximately 0.075 per cent, 1 gram; and if 0.05 per cent or less, 1.5 gram.

Adjust pH to 8.0–8.2. Boil to clarify, about thirty minutes. Determine pH and if less, adjust to 8.0 and boil three minutes more. Strain. Filter through paper, make up weight to 1015 grams, and filter through a filter press. Usually dispense 2500-ml. amounts in 4-liter bottles. Autoclave at 115°–118° for thirty minutes. If a sediment develops, *filter and redispense aseptically* as required and heat the material for ten to thirty minutes in steam at 100°. Incubate at 35°–37° for forty-eight hours and inspect.

F5A, A′. Beef-infusion broth (for streptococci and pneumococci): A. With glucose; A′. With buffered glucose

	A	*A′*
Beef	450	450 grams
Peptone (Difco proteose)	20	20 grams
Sodium chloride	5	0 grams
Glucose	0.5–1.5	0 grams
Water	1000	1000 grams

A and *A′*. Infuse the meat, strain, pass residue through meat press and use for *F1E*. Combine the juices and make up weight to 1 kg. Boil to clarify about thirty minutes, strain, filter through paper, make up weight, and *determine reducing sugar content*. Add the peptone and dissolve by stirring. *A*. Add the salt and the required amount of glucose and dissolve by stirring.

If the reducing sugar content of the infusion is equivalent to from 0.1 to 0.15 per cent glucose, add 0.5 gram glucose per kg.; if approximately 0.075 per cent, 1 gram; if approximately 0.05 per cent or less, 1.5 gram.

A and *A′*. Adjust pH to 8.2 and autoclave in bulk 20- to 40-kg. amounts thirty to forty minutes and make up weight of *A* to 1025 grams, of *A′* to 1020 grams. Determine pH. If less, adjust to 8.0 and boil three to five minutes. Filter through paper. Dispense as required and autoclave at 115°–118° for thirty minutes.

A. For cultural purposes additional glucose is usually unnecessary. For toxin production, however, add aseptically before inoculation 20-per-cent glucose solution (*F62*) as specified.

A′. Add aseptically a specified amount, usually 5 ml. per 100 ml., of buffered glucose solution, prepared as follows:

Buffered glucose solution

Sodium bicarbonate, $NaHCO_3$	4 grams
Disodium phosphate, Na_2HPO_4	0.8 gram
Sodium chloride	4 grams
Glucose	15 grams
Water to make	100 ml.

Make a solution, dilute to volume, and sterilize by filtration.

F5B. Beef-infusion broth, sugar-free, plus carbohydrate and indicator

Beef infusion, concentrated, sugar-free, *F1C*	500 grams
Water	500 grams
Peptone (Difco proteose)	20 grams
Sodium chloride	5 grams
Chlorophenol red, 0.04-per-cent solution	2 ml. per 100 ml.
Carbohydrate, 10-per-cent solution, *F62*	10 ml. per 100 ml.

To the infusion add the water, peptone, and salt. Heat if necessary to dissolve. Adjust pH to 8.0. Autoclave in bulk for ten minutes. Make up total weight, 1025 grams, and filter through paper. Dispense as ordered, usually 100 ml. in 500-ml. flasks. Autoclave twenty minutes. Aseptically add the chlorophenol red solution and the specified carbohydrate solution. Heat at 100° for ten minutes and dispense 2.5-ml. amounts in 11- by 75-mm. tubes. Trim and paraffin plugs. Incubate forty-eight hours and inspect.

F5.5. Ten-per-cent meat-infusion broth (for streptococci and pneumococci)

Beef or beef heart, as specified	100 grams
Peptone (Difco proteose)	20 grams
Sodium chloride	5 grams
Dipotassium phosphate, K_2HPO_4	1 gram
Monopotassium phosphate, KH_2PO_4	1 gram
Glucose	1–2 grams
Water to make	1000 grams
Calcium chloride, $CaCl_2$, 10-per-cent solution (*F61*)	2 ml.

Infuse the meat with 250 grams of water, strain, and pass residue through meat press, and determine the reducing sugar content of the meat juice. Dissolve the peptone, salt, phosphates, and the required amount of glucose in water, combine with the meat juices and make up to 1 kg.

If the reducing sugar content of the meat juices is the equivalent of from 0.125 to 0.15-per-cent glucose, add 1 gram glucose; if between 0.075 and 0.1 per cent, add 1.5 gram; if less than 0.05 per cent, from 1.75 to 2.0 grams.

Adjust pH to 8.0–8.2. Add the calcium chloride solution. Autoclave in bulk thirty minutes and make up weight. Determine pH and if less, adjust to 7.8–8.0 and boil three to five minutes. Filter through paper. Dispense as ordered. Autoclave at 115°–118° for thirty minutes.

F6. Beef-infusion broth with chopped meat (2) (for anaerobes)

Beef	900 grams
Water	1000 grams

Infuse, boil twenty minutes, make up weight to 1.9 kg., cool, and remove fat. Filter through paper a small sample of the liquor and titrate 5 ml. to pH 8.0, using 0.05 N NaOH, of which let x ml. be the required amount for the 5-ml. sample of juice. Then admix $20x$ ml. of N NaOH with the infusion mixture. Repeat the operation to adjust pH to 8.0. Practically, this amounts to the approach method of adjustment, although it would appear that the alkali added each time is twice that ordinarily required in adjustments of liquor alone. Decant most of the liquor. Dispense the chopped meat in 16- by 133-mm. tubes enough to fill the tubes one-third and add enough of the decanted liquor to leave a margin of coverage about 25 mm. Cover cotton plug with Cellophane. Autoclave thirty minutes. Incubate forty-eight to seventy-two hours at 35°–37°. Inspect.

F7. Beef-infusion broth with glucose and blood (3)

Beef-infusion broth (*F2A* or *F4*)	100 ml.
Glucose, 20-per-cent solution (*F62A*)	5 ml.
Rabbit blood, defibrinated, sterile	5 ml.

With aseptic precautions, combine the sterile glucose solution, blood, and beef-infusion broth. Mix well and dispense aseptically 5-ml. amounts in 15-ml. centrifuge tubes. Incubate a few tubes forty-eight hours at 35°–37° and inspect.

F8. Beef-infusion broth with glycerol (4) (for tubercle antigen and tuberculin)

Beef	450 grams
Peptone (Fairchild's or equal)	10 grams
Sodium chloride	5 grams
Glycerol	40 ml.
Water	1000 grams

Infuse the meat overnight at 4°–6° and then at 45°–50° for thirty minutes with occasional stirring. Boil five minutes, stirring frequently. Strain, remove fat, and make up weight to 1 kg. Add the peptone and salt, dissolve, and adjust pH to 7.6. Add the glycerol, autoclave five minutes, and make up weight to 1065 grams. Adjust pH to 7.6, boil five minutes, and make up weight. Filter through paper. Usually dispense 125-ml. amounts in 1-liter flat-sided bottles, and sterilize fractionally for three 30-minute periods, incubating six hours at 35°–37° between successive treatments. Subsequently incubate seventy-two to ninety-six hours at 35°–37°. Inspect.

F9. Beef-infusion egg medium (5) (for Mycobacterium tuberculosis)

Beef	225 grams
Glycerol	37.5 ml.
Water	212.5 ml.
Eggs	6
Crystal violet, if specified, 1-per-cent alcoholic solution	1 ml. per 100 ml.

The day before needed prepare certain apparatus as follows:

Line a 2-liter pail with two layers of gauze leaving an overhang of about 8 cm. Fit the metal cover in place. Wrap a large spoon in heavy brown paper and prepare a beaded 2-liter flask. In a 500-ml. flask mix the glycerol and water and autoclave with the prepared apparatus for thirty minutes. The following day, dissect the required amount of meat from within a considerably larger portion, using a cutting board and knife disinfected by boiling water. Disinfect a meat grinder similarly, transfer the meat on the knife, and grind into the sterile gauze in the pail previously prepared. Add the glycerol solution, cover, and infuse overnight. Gather the meat in the gauze twisted to form a bag, and press against the side of the pail with the sterile spoon. Repeatedly twist and squeeze to expel the juice and then discard the meat. Weigh the beaded flask, aseptically transfer the egg contents, and determine the increase in weight. Admix aseptically 0.5 ml. of the infusion per gram of egg contents. If required, admix 1 ml. of 1-per-cent alcoholic solution of crystal violet per 100 ml. of the previous mixture. *Attach a sterile drain with strainer* and dispense aseptically as required. Inspissate at 80°–85° until all the medium has solidified, and on two succeeding days heat at about 75° for one hour. Trim the plugs and seal with paraffin. Incubate forty-eight hours at 35°–37°, and inspect.

Crystal violet, alcoholic solution, 1-per-cent

Crystal violet	1 gram
Alcohol, 95-per-cent, to make	100 ml.

Dissolve the dye in part of the alcohol and make up to volume. Dispense in a glass-stoppered bottle and store.

F10. Beef-heart-infusion broth (6) (for Neisseria gonorrhoeae)

Beef heart	450	grams
Peptone	10	grams
Sodium chloride	5	grams
Egg	1	
Water, sterile distilled	1000	grams
Glucose, 20-per-cent solution (*F62A*)	37.5 ml. per liter	

Obtain hearts carefully dissected from the animal without rupture of the pericardial sac. Remove them from the sacs with care to avoid contamination. Trim and grind as described in *F9*, and collect the ground meat in a sterilized container. Add the water and transfer the egg contents aseptically. Heat at 60° for five minutes stirring constantly with a sterile glass rod. Chill and remove fat with a flamed wire-mesh strainer. Add the peptone and salt and heat five minutes at 65°–70° with constant stirring. Adjust pH to 6.6–6.8 with N Na_2CO_3. Heat in bulk in steam at 100° for one hour, loosen the clot, and heat again for one hour. Strain through a colander and filter through glass wool. Weigh the filtrate and adjust pH to 7.2 with N Na_2CO_3. Heat in bulk in steam at 100° for fifteen minutes. Dispense 1200-ml. amounts in 2-liter flasks and sterilize fractionally for three 30-minute periods. Store four days at 20°–27°. Inspect and store. As required, add aseptically 45 ml. of 20-per-cent glucose solution (*F62A*) to each 1200-ml. amount of the prepared broth, attach a sterile drain, and dispense aseptically as ordered. Heat in steam at 100° for ten minutes, incubate forty-eight hours at 35°–37° and inspect.

F11. Veal-infusion broth (7, 8) (for botulinus toxin)

Veal	900 grams
Peptone (Eimer and Amend)	10 grams
Sodium chloride	5 grams
Water	1000 grams
Glucose, 20-per-cent solution (*F62A*)	100 ml. per liter

Infuse the meat with nine-tenths of the water. Strain. Pass residue through a meat press and use in *F1D*. Combine juices, and make up weight to 900 grams. Boil to clarify, strain, add the peptone and salt dissolved in the remaining water, and make up weight to 1015 grams. Adjust pH to 8.4, boil five minutes, and make up weight. Filter through paper until clear. Dispense in 1-liter flasks for toxin production, and in 22- by 175-mm. Pyrex test tubes for seed cultures. Sterilize fractionally, three 30-minute periods.

NOTE: Dispense in amounts as great as possible with due allowance for glucose and culture additions to be made without danger of wetting the plugs. Just before inoculation, aseptically add 10 ml. of sterile 20-per-cent glucose solution (*F62A*) per 100 ml. of medium.

F12. Veal-infusion broth (9) (for diphtheria toxin)

Veal	450 grams
Peptone (Difco proteose)	20 grams
Sodium chloride	5 grams
Water	1000 grams
Glucose (Bacto), 20-per-cent solution (*F62A*)	10 ml. per liter

Infuse the meat, strain, pass residue through meat press and use in *F1E*. Combine juices, and make up weight to 1 kg. Adjust pH to 6.8–7.0. Warm to 35°–37° and add 10 ml. of an 18- to 24-hour broth culture of *Bact. coli*. Stir well and incubate at 35°–37° overnight. If the odor developed by the following morning is not intense, it may be advisable to allow the action to continue for twenty-four hours longer. Again adjust pH to 6.8–7.0. Heat in steam at 100° about one hour or more. Strain, add the peptone and salt, stir well to dissolve, and make up weight to 1025 grams. Adjust pH to 7.6–7.7, boil five minutes, make up weight, and filter through paper. Dispense 500-ml. amounts in 2-liter flasks and sterilize fractionally for three 30-minute periods. Just before inoculation add aseptically to each flask 5 ml. of a sterile 20-per-cent glucose solution (*F62A*).

NOTE: For maintenance of stock cultures and for seed tubes, before sterilization add 10 ml. of 20-per cent glucose per liter of broth, dispense 2-ml. amounts in 13- by 125-mm. test tubes and sterilize fractionally for three 30-minute periods.

F13.1: A, B. Meat-infusion broth with hog's stomach autolyzate (Martin's peptone (10, 11); B. With reduced iron (12) (for tetanus toxin production)

	A	*B*	
Veal (or beef heart)	225	225	grams
Water	500	500	grams
Hog's stomach autolyzate	500	500	grams
Calcium chloride, $CaCl_2$, 25-per-cent solution (*F61B*)	2.5	2.5	ml.

	A	*B*
Sodium chloride	2.5	2.5 grams per kg.
Glucose	7.5	7.5 grams per kg.
Nicotinic acid, 0.1-per-cent solution, freshly prepared	0.2	0.2 ml. per kg.
Reduced iron (Merck) metal, as specified	0	0.2–0.5 grams per 1600 ml.

A, *B*. Infuse the meat overnight at 4°–6°, then for thirty minutes at 45° with stirring. Boil fifteen to twenty minutes to clarify. Strain, chill, remove fat, and filter through paper. Store at 4°–6° overnight. To 500 grams of hog's stomach autolyzate, add the required amount of calcium chloride. Heat to approximately 60° and combine with the meat infusion separately warmed to 60°. Heat to 80° and adjust to pH 7.8–8.0 with approximately 40-per-cent sodium hydroxide.

A. Remove a 50-ml. sample, clarify by filtration through No. 3 Whatman filter paper or by centrifugation and *determine the iron concentration.* If the concentration is greater than the equivalent of 0.05 mg. Fe. per liter, add per liter 1 ml. of 25-per-cent $CaCl_2$ to the bulk material; readjust pH to 8.0 if necessary, let stand at 80° for ten minutes, remove a second sample and *determine the iron concentration.* (A second addition of $CaCl_2$ is seldom necessary.)

When the iron concentration of the test sample is between the equivalent of 0.025 and 0.05 mg. Fe per liter, filter the bulk of the material through paper until clear.

B. Filter through paper until clear.

A, *B*. Weigh the filtrate. Add the required amount of sodium chloride and glucose and dissolve by stirring. Adjust pH to 7.2 and add the nicotinic acid.

To prepare the nicotinic acid solution, weigh 0.01 gram of the solid in a 50-ml. beaker, add 0.05–0.10 ml. of *N* HCl to dissolve and dilute with 9.9 ml. of water.

A. Dispense 1600-ml. amounts in 2-liter flasks. *B*. Dispense 1600-ml. amounts in 2-liter flasks to which the specified amount of the reduced iron has been added. *A*, *B*. Heat in steam at 100° for fifteen minutes, then autoclave at 115°–118° for thirty minutes. Remove the flasks from the autoclave as soon as possible and chill in running cold water. Let stand at room temperature overnight before inoculating.

NOTE: For seed cultures for the inoculation of flasks of medium *A*, prepare two media as follows:

1. Place cooked meat residue from original infusion in 16- by 150-mm. Pyrex test tubes to a depth of not more than 25 ml. and add 6–8 ml. of medium *A* to each tube. Autoclave at 115°–118° for twenty minutes.

2. To 200 ml. of freshly prepared basal medium *A*, add 0.1 ml. of a 0.1-per-cent $FeSO_4 \cdot 7H_2O$ solution. Mix thoroughly and dispense approximately 25-ml. amounts in 22- by 175-mm. Pyrex test tubes. Autoclave at 115°–118° for twenty minutes.

Hog's stomach autolyzate (Martin's peptone)

Hog's stomach	350	grams
Water	1000	ml.
Hydrochloric acid, concentrated (sp. gr. 1.18)	8.66	ml.

Usually prepare about 30 liters of autolyzate. Twenty stomachs from freshly killed hogs should be obtained directly from the slaughter house where they should have been rinsed on the outside with cold water and promptly packed in ice for transport to the laboratory. Upon receipt at the laboratory, cut open the stomachs and rinse out the contents with cold water. Trim fat from the outsides as thoroughly as possible with scissors. If it is not possible to start the digestion on the same day the stomachs are received, store in a covered enamel container overnight at 4°–6° or up to three or four days at −5° to −7°.

Pass the stomachs through a fine grinder. To each of several 6-liter amounts of water, warmed to 49°, add 52 ml. of concentrated hydrochloric acid, then 2100 grams of the ground stomachs and mix thoroughly. Transfer to 9-liter Pyrex bottles and place in a water bath regulated to 45°–47°. Maintain the temperature of the mixture inside the bottles at about 46° for twenty to twenty-two hours. Stir at frequent intervals during the day and night. At the end of the digestion period, raise the temperature of the mixture in the bath as rapidly as practicable to 85°. Remove the bottles from the bath, place in running water and cool the contents to 30°, then let stand at 4°–6° for twenty-four to forty-eight hours to allow the sludge to settle. Let stand for one to two hours at room temperature. Siphon off the supernatant and filter through paper pulp or wet pads of absorbent cotton. Have the pH of the filtered autolyzate determined electrometrically; it should be 3.2 to 3.4. Store in 4-liter bottles at 4°–6°. If not to be used within twenty-four to forty-eight hours, add chloroform as a preservative. Ordinarily, it should not be used after three to four weeks.

F13.1: C. Meat-infusion broth (12) (for maintenance of cultures of Clostridium tetani)

Meat infusion	1000 grams
Sodium chloride	5 grams
Peptone	10 grams
Glucose	10 grams

To 1000 grams of the meat infusion (of *F13.1*) before boiling, add the peptone, salt, and glucose. Adjust pH to 7.8–8.0, boil five to ten minutes to clarify, make up weight and filter through paper. Dispense 8-ml. amounts in 16- by 133-mm. test tubes. Autoclave at 115°–118° for ten minutes. After sterilization, the pH should be 7.2–7.4.

F14: A, B, C. Beef-extract broth: A. With sodium chloride; B. With carbohydrate (13a); C. With carbohydrate double strength

	A	*B*	*C*
Beef extract	3	3	6 grams
Peptone	5	5	10 grams
Sodium chloride	5	0	0 grams
Carbohydrate, as specified	0	5	10 grams
Water	1000	1000	1000 grams

Combine all but the carbohydrate, heat to 65°, and stir to dissolve. Adjust pH to 7.0. (The pH after final sterilization should be 6.4–7.0, preferably 6.9.) Bring to a boil and make up weight. (Lots of less than 5 kg. should be cooled to 25° before adjusting weight.) Then add the carbohydrate, if required, and dissolve with stirring. Filter through paper.

A. Dispense as ordered and autoclave twenty minutes. *B*. Dispense in small double-tube fermentation sets and autoclave twelve minutes. *C*. Dispense in large double-tube fermentation sets precise amounts to yield 8 ml. ±0.2 ml. of medium per set after sterilization. Autoclave twelve minutes.

F15. Coagulated serum medium, Loeffler (14)

Beef extract	3 grams
Peptone	10 grams
Sodium chloride	5 grams
Glucose	10 grams
Water	1000 ml.
Horse serum	3000 ml.

Combine the beef extract, peptone, sodium chloride, glucose, and water, and dissolve with heat. Cool mixture to 55°. Admix the serum and filter the mixture through infusorial earth in a Büchner funnel. Sterilize by filtration and dispense aseptically 4000-ml. amounts in 4-liter bottles and store. When required, dispense aseptically 2- to 3-ml. amounts in 13- by 125-mm. test tubes with combination cotton-paraffined-cork plugs. Do not flame tubes and avoid formation of bubbles. Slant in metal boxes on an inclined rack in layers with narrow strips of paper between to retain the desired inclination. Coagulate the medium by a special procedure using steam under pressure with entrapped air (see chapter on "Sterilization," p. 242). On removing from the sterilizer, push corks in firmly, incubate twenty-four hours at 35°–37° and forty-eight hours at 20°–27°. Seal tubes by dipping corked ends into melted paraffin and store at 20°–27°.

F15.6. Egg medium (15) (for maintenance of Vi strains of Bacterium typhosum)

Eggs	(6) 300 grams
Sodium chloride, 0.5-per-cent solution, sterile	100 ml.

Autoclave the sodium chloride solution for twenty minutes. Cool. Transfer the egg contents aseptically to a weighed sterile beaded flask, and, per 300 grams of egg, add aseptically 100 ml. of sterile salt solution. Mix by shaking vigorously. Dispense aseptically through a *sterile drain with strainer* 3-ml. amounts in 13- by 125-mm. test tubes with special combination cotton-cork plugs. Slant and inspissate at 80° for one hour on two successive days. Incubate forty-eight hours at 35°–37°. Inspect.

F16. Egg albumen medium (16) (for Endamoeba histolytica)

Eggs	10	
Locke's solution (*F63*)	125	ml.
Egg albumen	8.75	grams
Locke's solution (*F63*)	875	ml.

Autoclave 125 ml. of Locke's solution thirty minutes in a 3-liter beaded flask. Cool. To this transfer the egg contents aseptically. Mix by shaking vigorously. Dispense aseptically 6-ml. amounts in 16- by 133-mm. test tubes and slant to give a butt about 2 cm. deep and slant about 5 cm. long. Inspissate at 80° for one hour on two successive days. Dissolve the egg albumen in the second portion of Locke's solution with stirring, sterilize by filtration, and aseptically add enough to each of the prepared slants to rise 1 cm. above the solid medium with the tube upright. Incubate forty-eight hours at 35°–37° and inspect.

F17. Egg malachite green medium (17, 18) (for Mycobacterium tuberculosis)

Potassium sulfate, K_2SO_4	1.2	grams
Magnesium sulfate, $MgSO_4$	0.12	gram
Magnesium citrate, $Mg_3(C_6H_5O_7)_2$	0.3	gram
Asparagin	1.8	grams
Glycerol	6	ml.
Water to make	300	ml.
Potato starch (Eimer and Amend)	15	grams
Eggs	10	
Malachite green, 2-per-cent solution	10	ml.

Make a solution of the first group of ingredients by stirring together in a beaded flask, heat two hours in steam at 100°. Store. As required, place in a boiling water bath and admix the starch with continual stirring for fifteen to twenty minutes until a smooth viscous, barely fluid paste is formed. Allow to stand one hour in a bath at 54°–56°. Aseptically add the egg contents. Mix thoroughly and admix the malachite green. Dispense aseptically through a *sterile drain with strainer* in tubes or jars as rapidly as practicable, avoiding adherence on the sides. Slant, heat slowly to 50° in autoclave and hold for thirty minutes; then raise temperature slowly to 90°–95° and hold for one hour. See chapter on "Sterilization," p. 242. Incubate for twenty-four hours at 35°–37°. Inspect.

NOTE: The product in cotton-plugged tubes should remain satisfactory stored at 4°–6° for a month and in screw-capped jars it should keep four months.

F17.2. Egg yolk potato medium (19) (for Mycobacterium tuberculosis)

Whole egg	1
Egg yolks	11
Malachite green, 2-per-cent solution, sterile	1 ml. per 100 grams
Potato base	100 ml. per 100 grams

Aseptically transfer the egg yolks and the contents of the whole egg into a weighed sterile beaded flask. Per 100 grams of egg mixture add aseptically 1 ml. of malachite green solution, then 100 ml. of the potato base. Mix thoroughly and dispense aseptically through a *sterile drain with strainer*. Slant in metal boxes on an inclined rack 5- to 6-ml. amounts in tubes or jars with screwcaps. Heat slowly to 50° in the autoclave using steam under pressure with entrapped air (see chapter on "Sterilization," p. 242) and hold for thirty minutes; then raise temperature slowly to 90°–95° and hold for one hour.

Potato base

Potatoes, peeled ground	200	grams
Citric acid, $H_3C_6H_5O_7 \cdot H_2O$ (A.C.S.)	0.2	gram
Blood powder	5	grams
Glycerol	60	ml.
Water to make	1000	grams

Peel the potatoes, pass through a fine grinder, and weigh. Mix all the ingredients and autoclave in bulk for thirty minutes. Filter through gauze. Dispense as required and autoclave thirty minutes. Store.

To prepare the blood powder, thoroughly dry guinea pig blood clots at 55°–60°. Grind the dehydrated clot to a fine powder and store.

F18:A, B. Skim milk; B. With indicator

Place about 930 ml. of certified or grade A milk in a separatory funnel and store at 4°–6° overnight. Draw off the fat-free layer. *A*. Dispense as required and sterilize fractionally for three 20-minute periods. *B*. Add 1 ml. of 1.6-per-cent bromocresol purple (95-per-cent alcohol solution) or other suitable indicator, per liter of skim milk. Dispense as required and sterilize fractionally for three 20-minute periods.

F19. Peptone medium with glucose (13b)

Peptone (Difco proteose)	5 grams
Dipotassium phosphate, K_2HPO_4	5 grams
Water to make	1000 ml.
Glucose, 20-per-cent solution, sterile (*F62A*)	25 ml.

In eight-tenths of the water dissolve the phosphate and peptone by heating on a water bath for twenty minutes with occasional stirring. Filter through paper, cool to 20°, and make up to 1 liter. Dispense as required. Autoclave twenty minutes. Store. Aseptically add the glucose. Aseptically dispense as ordered in 13- by 125-mm. test tubes and heat in steam at 100° for ten minutes. Incubate for forty-eight to seventy-two hours at 35°–37°. Inspect.

F20. Peptone medium, infusion-free (20) (for diphtheria toxin)

Sodium chloride	2.5	grams
Dipotassium phosphate, K_2HPO_4	1	gram
Monopotassium phosphate, KH_2PO_4	1	gram
Peptone (Difco proteose)	20	grams
Calcium chloride, $CaCl_2$, 10-per-cent solution (*F61*)	1.25	ml.
Water to make	500	grams
Water		kg. per kg.
Magnesium sulfate, $MgSO_4$	0.2	grams per kg.
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$, 0.1-per-cent solution, freshly prepared	0.3–0.5	ml. per kg.

The optimum amount of iron required may vary with different lots of peptone or other ingredients in the basal medium or in the adjuvant solutions I and II below. It should be determined by tests for toxin production in small experimental batches.

Dissolve the sodium chloride, phosphates, and peptone in part of the water. Admix the $CaCl_2$, make up to 500 grams, and adjust pH to 7.6–7.8. Hold at 80°–90° for twenty to thirty minutes. Filter through paper. To the filtrate recovered, add an equal weight of water. Admix the magnesium sulfate and the iron solution.

Adjust pH to 7.2. For seed tubes, add 0.2 gram of glucose per 100 ml. of basal medium and dispense 2-ml. amounts in 18- by 125-mm. Pyrex tubes. For toxin production dispense as specified usually 190 or 285 ml. in 2-liter Erlenmeyer flasks or 285 ml. in 2-liter Pyrex diphtheria toxin bottles. Sterilize at 115°–118° for fifteen minutes.

At the time of inoculation approximately per 95 ml. of medium, add aseptically 5 ml. of solution I or II as specified.

Solution I

Sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$ (A.C.S., iron less than 0.00002 per cent), as specified	20–30	grams
Maltose, as specified	16–25	grams
Glucose, as specified	0–1	gram
Calcium chloride, $CaCl_2$, 10-per-cent solution (*F61*)	1.5	ml.
Water to make	100	ml.

Dissolve the sodium acetate in not more than 30 ml. of warm water, admix the other ingredients and make up to 100 ml. Sterilize by filtration and test for sterility at least in duplicate in mixtures with the basal medium using 2 ml. of solution per 50 ml. of medium. Dispense aseptically as specified.

Solution II

Sodium lactate	15	grams
Maltose	15	grams
Glucose, as specified	0–1	gram
Calcium chloride, $CaCl_2$, 10-per-cent solution (*F61*)	1.5	ml.
Water to make	100	ml.

To 11.8 ml. of lactic acid (85-per-cent by weight), add rapidly about 12 ml. of 10 *N* NaOH (approximate 40-per-cent solution). Then while still hot continue adding NaOH until pH is about 7.0 as indicated in side spot-tests with phenol red. The solution should then contain 15 grams of sodium lactate. Cool, admix the carbohydrates and calcium chloride and make up to 100 ml. Sterilize and dispense as indicated for Solution I.

F21: A, B, C. Peptone medium, infusion-free (21): A. With sugar; B. With buffered sugar (for pneumococci and streptococci); C. With glucose (for streptococci)

	A, B	*C*
Sodium chloride	5	5 grams
Dipotassium phosphate, K_2HPO_4	1	1 gram
Monopotassium phosphate, KH_2PO_4	1	1 gram
Peptone (Parke, Davis) as specified	10 or 20	0 grams
Peptone (Wilson's thio or Nutri) as specified	0	20 grams
Tap water to make	1	1 kg.

Dissolve the salts and peptone in part of the water and make up weight to 1 kg. Adjust pH to 7.8. Autoclave in bulk forty minutes.

A. Add sugar, beet or cane, as specified by kind and amount (usually 2 or 7.5 grams), and make up weight to 1 kg. Filter through paper. Dispense and autoclave as ordered:

6 ml. in tubes (16 by 133 mm.)	autoclave fifteen minutes
100 ml. in 200-ml. French square bottles	autoclave fifteen minutes
450 ml. in 1-liter bottles	autoclave thirty minutes
3500 ml. in 4-liter bottles	autoclave thirty minutes

B. Make up weight, filter through paper, and dispense in 2-liter amounts or more as required. Autoclave thirty minutes. Add aseptically a specified amount of the specified buffered sugar solution (usually 5 ml. per hundred), dispense as ordered, and heat in steam at 100° for ten minutes. Incubate forty-eight hours at 35°–37° and inspect.

C. Add 7.5 grams glucose and make up weight to 1 kg. Filter through paper. Dispense as specified and autoclave twenty minutes.

Buffered beet or cane sugar solution, 6-, 12-, or 15-per-cent, as specified

Sodium bicarbonate, $NaHCO_3$	40 grams
Disodium phosphate, Na_2HPO_4	8 grams
Sugar, commercial (beet or cane as specified)	60, 120, or 150 grams as specified
Tap water to make	1000 ml.

Combine all ingredients, stir to dissolve, and make up to volume. Dispense in 100-ml. amounts in 200-ml. French square bottles. Sterilize fractionally for three 20-minute periods.

F22. Peptone medium with indicator and carbohydrate (22)

Peptone (Parke, Davis)	10 grams
Sodium chloride	5 grams
Andrade's indicator (of *F50*)	10 ml.
Water	1000 grams

Carbohydrate (biologically tested) as specified (*F62*)

Combine peptone, salt, and water, and dissolve with heat. Filter through paper, add the indicator, and adjust pH to 7.4–7.5. Dispense as required and autoclave twenty minutes. Store. Per 100 ml. add aseptically 1 gram of the specified carbohydrate in a sterile prepared solution (*F62*) and mix well. Dispense aseptically 2-ml. amounts in 11- by 75-mm. tubes; incubate forty-eight hours at 35°–37°. Inspect, trim plugs and seal with paraffin, and store.

F22.5. Peptone medium with honey (23)

Peptone	10 grams
Honey	60 grams
Water	1000 grams

Dissolve the peptone and honey in the water and adjust pH to 5.6 with 10-per-cent tartaric acid, using methyl red as the indicator. Dispense as required and autoclave fifteen minutes.

F23: A, B. Peptone bile medium (24)

	A	*B*
Peptone	10	10 grams
Ox bile, fresh	30	0 grams
Ox bile, dehydrated	0	5 grams
Water	1000	1000 grams

Combine the bile, peptone, and water, boil vigorously for three to five minutes, dispense in about 2000-ml. amounts in 3-liter flasks, and store at 4°–6° overnight. Filter through hard paper. Redispense in 600-ml. amounts in 1-liter flasks and autoclave twenty minutes. Store. When required, filter through hard paper, dispense 7-ml. amounts in 16- by 150-mm. tubes and autoclave twenty minutes.

F24. Peptone bile medium with brilliant green and lactose (13c)

Peptone	10	grams
Lactose	10	grams
Ox bile, dehydrated	20	grams
Brilliant green, 0.1-per-cent solution	13.3	ml.
Water to make	1000	ml.

Dissolve the peptone and lactose in 500 ml. of water, and the ox bile (pH 7.0–7.5) in 200 ml. of water. Mix the two solutions and make up to 975 ml. Adjust pH to 7.4. Add the brilliant green and make up to 1000 ml. Filter through cotton. Dispense 10-ml. amounts in small double-tube fermentation sets and autoclave twelve minutes.

Brilliant green solution, 0.1-per-cent

Mix 0.5 gram of the dye (Coleman and Bell) with water, dilute to 500 ml., let stand overnight, mix thoroughly, filter through paper, and dispense in glass-stoppered bottles. Store in the dark.

F25. Peptone bile glycerol medium (25)

Ox bile, dehydrated	100 grams
Glycerol	100 ml.
Peptone	20 grams
Water to make	1000 ml.

Dissolve the ingredients in part of the water and make up to volume. Filter through paper. Dispense in 8.5-ml. amounts in 65- by 35-mm. jars, cap loosely, and autoclave fifteen minutes. Let cool to about 45° and fasten caps securely.

F25.5. Peptone tetrathionate medium (26)

Peptone (Difco proteose No. 3)	5	grams
Bile salts (Bacto No. 3)	1	gram
Water	1000	grams
Calcium carbonate, $CaCO_3$	10	grams per liter
Sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$ (A.C.S.)	30	grams per liter
Brilliant green, 0.1-per-cent solution (of *F24*)	11	ml. per liter
Aqueous iodine, 25-per-cent solution	2.5	ml. per 100 ml.

Dissolve the peptone and bile salts in the water and dispense 500-ml. amounts in 1-liter flasks. Add the calcium carbonate to each flask and autoclave twenty minutes. Store. As required, add the sodium thiosulfate and brilliant green and dispense aseptically 12-ml. amounts in 65- by 35-mm. jars. To keep the suspension of calcium carbonate uniform, shake the flask before filling the first jar and after filling every third succeeding jar. When the medium is used, the required amount of the iodine solution is added aseptically to each jar (p. 298).

Aqueous iodine solution, 25-per-cent

Iodine	25 grams
Potassium iodide, KI	20 grams
Water to make	100 ml.

Dissolve the iodine and potassium iodide in part of the water and make up to volume.

F26. Peptone-dialyzate medium (27, 28) (for pneumococci)

Peptone-dialyzate solution	500 grams
Sodium chloride	5 grams
Dipotassium phosphate, K_2HPO_4	1 gram
Monopotassium phosphate, KH_2PO_4	1 gram
Tap water to make	1000 grams
Carbohydrate if and as specified	

Dissolve the salts in part of the tap water, combine with the peptone-dialyzate solution, and make up weight to 1 kg. with tap water. Adjust pH to 7.7–7.8. Autoclave in bulk for forty minutes, add carbohydrate if and as specified, and make up weight. Filter through paper. Usually dispense 3200-ml. amounts in 4-liter bottles and autoclave thirty minutes; 100-ml. amounts in 200-ml. bottles and autoclave fifteen minutes.

Peptone-dialyzate solution

Peptone (Wilson's Nutri)	1160 grams
Tap water to make	4500 grams
Tap water	24500 grams

To the peptone add hot tap water to make 4.5 kg., and stir to dissolve. Place half in each of two Cellophane tubes (about 114 mm. diameter, 840 mm. length, and 0.089 mm. wall-thickness), one end of each tube being tied with heavy twine. Tie the other ends similarly, holding the tubes at all times so that liquid cannot spill or seep inadvertently through the ends. Immerse in the 24.5 kg. of water and adjust the height if necessary during dialysis so as to maintain approximate equality of liquid-level inside and outside the tubes. This water should be warm, 50°–70°. Heat rapidly to 90° and hold there for seven hours. Remove the tubes and contents, and store the dialyzate at 4°–6° overnight.

F27. Potato medium

To make 50 slants wash and peel about 8 large, sound potatoes, and cut out the eyes. With a cork-borer cut out cylinders of the proper size to fit the tubes to be used. Cut the cylinders in about 75-mm. lengths. Slice each piece diagonally in half to give two good slants. Wash in running tap water overnight. Place slants in tubes, plug, and cover with Cellophane. Place the tubes in quart Mason jars containing about a 25-mm. depth of mercuric chloride (1 g.p.l.) and autoclave twelve minutes. Seal jars and store.

F27.5. Rice medium (29)

Rice, white grains	7 grams
Water	28 grams

Place rice and water in 300-ml. flasks; with rice grains evenly distributed over bottom of flask set evenly in the sterilizer and autoclave twenty minutes.

F28. Serum water with indicator and carbohydrate (22, 30)

Horse serum	200 ml.
Water	800 ml.
Andrade's indicator (of *F50*)	10 ml.

Carbohydrate (biologically tested) (*F62*), as specified

Admix the serum with the water and heat in steam at 100° for twenty minutes. Admix the indicator, cool to 25°–30°, and filter through paper. Adjust pH to 7.0–7.2 with *N* HCl, being careful to add the reagent drop by drop, with vigorous stirring. Dispense as required and autoclave twenty minutes. Store. Per 100 ml. add aseptically one gram of the specified carbohydrate in a sterile prepared solution (*F62*) and mix well. Dispense aseptically 2-ml. amounts in 11- by 75-mm. tubes, incubate forty-eight hours at 35°–37°. Inspect, trim plugs and seal with paraffin, and store.

F29: A, B. Tryptone medium (31) (for indole test); B. With nitrate (for nitrosoindole reaction)

	A	*B*	
Tryptone	10	10	grams
Potassium nitrate, KNO_3	0	0.1	gram
Water	1000	1000	grams

Dissolve the tryptone in the water with heat if necessary, adjust pH (*A* to 7.0, *B* to 9.0–9.4), (*B*, admix the nitrate), and filter through paper. Dispense 3.5 ml. in 13- by 125-mm. tubes and autoclave twelve minutes.

F29.2. Casein hydrolyzate medium with yeast dialyzate (for Hemophilus pertussis vaccine preparation) (31a, 31b)

Casamino acid (Bacto)	10 grams
Sodium chloride (A.C.S.)	2.5 grams
Monopotassium phosphate, KH_2PO_4 (A.C.S.)	0.5 gram
Magnesium chloride, $MgCl_2 \cdot 6H_2O$ (A.C.S.)	0.4 gram
Starch, soluble, powdered	1.5 grams
Calcium chloride, $CaCl_2$ (A.C.S.), 1-per-cent solution	1 ml.
Ferrous sulfate, $FeSO_4 \cdot 7H_2O$ (A.C.S.), 0.5-per-cent solution	2 ml.
Copper sulfate, $CuSO_4 \cdot 5H_2O$ (A.C.S.), 0.05-per-cent solution	1 ml.
Cysteine hydrochloride, 1-per-cent solution	2.5 ml.
Yeast dialyzate	50 ml.
Distilled water to make	1 kg.

Either technical or purified casamino acid may be used. Selection of a suitable lot should be made by tests for growth in small experimental batches.

Dissolve the casamino acid, salt, phosphate, and magnesium chloride in part of the water. Add the starch, dissolved separately by heat in part of the water, then the remaining ingredients and make up to 1 kg. Adjust pH to 7.2–7.3. Filter and dispense in 200-ml. amounts in 1-liter Roux bottles. Autoclave at 10 lb. pressure for fifteen minutes.

Yeast dialyzate

Brewers' yeast (Fleischmann pure dry, type 2019)	500 grams
Distilled water	800 grams
Distilled water	2000 ml.

Transfer the dry yeast to a Cellophane tube (500–550 mm. length, about 114 mm. diameter, 0.089 mm. wall-thickness). Add the 800 grams of water and mix thoroughly by kneading gently. Tie each end of the tube with heavy twine, immerse it in the 2000 ml. of water in such a manner that the liquid cannot spill or seep through the ends of the tube, and adjust its position to give approximate equality of liquid-level inside and outside the tube. Heat at from 78° to 80° for seven hours. Remove the tube and transfer the dialyzate to a bottle. Add 5 ml. of chloroform per liter, stopper with a rubber stopper, and store in the coldroom. This dialyzate is clear, requires no filtration, and remains satisfactory for use even after several months' storage.

F29.4. Synthetic medium (20) (for Corynebacterium diphtheriae)

Distilled water, glass-distilled	1000	ml.
Sodium chloride (A.C.S.)	5	grams
Dipotassium phosphate, K_2HPO_4 (A.C.S.)	1.0	gram
Monopotassium phosphate, KH_2PO_4 (A.C.S.)	1.0	gram
Magnesium sulfate, $MgSO_4 \cdot 7H_2O$ (A.C.S.)	0.2	gram
Aspartic acid	3	grams
Sodium hydroxide, approximate 40-per-cent solution	2	ml.
Cystine (dissolved in 1- to 2-ml. *N* HCl) or Cysteine hydrochloride	0.5	gram
Sodium acetate, $NaC_2H_3O_2 \cdot 3H_2O$ (A.C.S.)	5	grams
Glucose	2	grams
Calcium chloride, $CaCl_2$ (A.C.S.), 0.5-per-cent solution (*F61A*)	2.5	ml.

None of the ingredients should contain more than a trace of iron salts. Dissolve the sodium chloride, phosphates, and magnesium sulfate in the distilled water, add the aspartic acid and concentrated sodium hydroxide, and heat to dissolve the aspartic acid. Add the cystine or cysteine hydrochloride, acetate, and glucose, and adjust the reaction to pH 7.6. Heat to boiling, then cool slightly before adding the calcium chloride. Filter through paper and dispense 2-ml. amounts in 16- by 125-mm. Pyrex test tubes. Sterilize at 115°–118° for twelve minutes. Each lot of medium is tested for ability to support growth before being used routinely and in general is not used after it is more than four to five weeks old.

Note: This medium is used for maintenance of the standard strain No. 3203 and for determining the nutritional requirements of other strains, especially those freshly isolated.

II. AGAR AND GELATIN MEDIA

F30. Beef-infusion gelatin (32)

Beef, ground	450 grams
Peptone	10 grams
Sodium chloride	5 grams
Gelatin	90 grams
Water	1000 grams

Infuse the meat overnight at 4°–6° and at 50°–55° for one hour. Strain and make up to 1000 grams. Adjust pH to 6.8–7.0. Add the other ingredients and dissolve with heat. Make up to total weight, filter through absorbent cotton in a Büchner funnel, weigh filtrate, and adjust pH to 7.8. Heat at 95°–100° for twenty minutes, stirring frequently. Filter through hot paper pulp in a Büchner funnel. Dispense 9-ml. amounts in 16- by 133-mm. tubes and autoclave at 115° for fifteen minutes. Trim and paraffin plugs.

F31. Beef-extract gelatin (131) (for liquefaction tests)

Beef extract	3 grams
Peptone	5 grams
Gelatin	120 grams
Water	1000 grams

Dissolve all ingredients with heat at 65°, make up weight, and adjust pH to 7.0. Heat, with vigorous stirring, until the mixture boils, make up weight, and filter through hot paper pulp. Dispense as required and autoclave fifteen minutes. The final pH should be 6.4–7.0. If the medium is to be employed for cultures incubated at 20°, *determine melting point*, which should be 25° or higher.

F32: A, B, C, D. Meat-infusion agar, semisolid (33, 34): A. Beef; B. Horse meat; C. Special, beef or horse meat; D. Special, with glucose (A and B for sterility tests; C for culturing blood; D for culturing blood for pneumococci)

	A	*B*	*C*	*D*	
Infusion broth (*F3A*)	1000	0	0	0	grams
Infusion broth (*F3B*)	0	1000	0	0	grams
Infusion broth (*F3C*)	0	0	1000	1000	grams
Agar	1.2	1.2	1.2	1.2	grams
Water	40	40	40	40	ml.
Glucose, 20-per-cent solution (*F62*)	0	0	0	3.75	ml. per 100 ml.
If specified,					
Sodium thioglycollate (34, 35)	0.5	0.5	0	0	grams per liter
Resazurin, 0.1-per-cent solution (freshly prepared) (35)	1.0	1.0	0	0	ml. per liter

	A	*B*	*C*	*D*	
Para-aminobenzoic acid or its sodium salt (36–38)	0	0	5	5	mg. per liter
Penicillin inactivator (39–45)	0	0	as specified		

A, *B*, *C*, and *D*. Use a fresh preparation of unsterilized broth. Dissolve the agar in the water by autoclaving and admix with the broth at 70°–80°. *A* and *B*. Filter through nonabsorbent cotton on two layers of soft filter paper in a Büchner funnel using a slight vacuum draft if necessary. Dispense as required and autoclave at 115°–118° for thirty minutes. When sodium thioglycollate and resazurin are specified, add to the filtered agar before dispensing.

Use a freshly prepared solution of resazurin. The sodium thioglycollate should be stored in a tightly stoppered bottle in a cool dry place and protected from light. Avoid contamination with water.

C and *D*. Filter hot through glass wool. Dispense 50-ml. amounts in 100-ml French square bottles with cotton and gauze plugs, or 80-ml. amounts in 100-ml. special blood-culture bottles with Bakelite screw cap and rubber diaphragm; cap loosely. Autoclave at 115°–118° for thirty minutes. Cool. *C*. Fasten screw caps securely and cover cap and neck with cellophane paper. *D*. Add aseptically to each bottle 3.75 ml. of a sterile 20-per-cent glucose solution (*F62*) per 100 ml. of medium and cap loosely. Cover cap and neck with Cellophane and heat in steam at 100° for fifteen minutes. Cool. Fasten caps securely and tie Cellophane cover firmly in place.

NOTE: When para-aminobenzoic acid is specified, it is added before sterilization; sterile penicillin inactivator is added just before use.

F33. Beef-infusion agar, residue

Beef-residue infusion (*F1D* or *E*)	1000	grams
Agar	17.5	grams
Sodium chloride	5	grams
Peptone	10	grams

Dissolve the agar in half the infusion by autoclaving, and the salt and peptone in the remainder. Combine. Make up to total weight. Adjust pH to 7.4–7.6, and filter through cotton. Dispense as required and autoclave twenty minutes.

F34: A, A′, B, B′. Beef-infusion agar: A. For vaccine preparation; A′. Sugar-free; B. Basal stock (for F34: C, D, E); B′. For Pasteurella pestis (46)

Beef infusion, concentrated (*F1B*)	500	grams
Agar	20	grams
Sodium chloride	5	grams
Peptone	10	grams
Water	500	grams

Dissolve the agar in the water by autoclaving, the salt and peptone in the infusion. Combine and make up to total weight. Adjust pH to 7.4–7.6.

A. Filter through cotton. Dispense 140-ml. amounts in 1-liter flat-sided bottles, autoclave thirty minutes, and slant. The final pH should be 7.2–7.4.

A′. If ordered sugar-free, substitute *F1C* for *F1B*.

B. Filter through cotton and gauze or by aspiration. Usually dispense 1-liter amounts in 2-liter flasks and autoclave thirty minutes.

B′. For *Past. pestis* use 30 instead of 5 grams of NaCl. Dispense as required.

F34C. Serum agar with glucose

Beef-infusion agar base, 2-per-cent (*F34B*)	1000 ml.
Horse serum, sterile	100 ml.
Glucose, 20-per-cent solution (*F62A*)	50 ml.

Melt the agar base *F34B* and cool to 50°. Warm the serum and glucose solution to 50°, combine aseptically with the melted agar, mix well, and dispense aseptically 7-ml. amounts in 19- by 150-mm. tubes. Slant, allow to solidify, incubate forty-eight hours at 35°–37°, then twenty-four hours at 20°–27°. Inspect and store.

F34D. Tissue semisolid agar (47, 48) (for Treponema)

Beef-infusion agar base, 2-per-cent (*F34B*)	100 ml.
Beef-infusion sterility broth (*F3A*)	233 ml.
Ascitic fluid, sterile	66 ml.
Normal rabbit kidneys, sterile	2

Combine the melted agar base *F34B* and broth in a 500-ml. flask and adjust pH to 7.2–7.4. Autoclave for twenty minutes. Exsanguinate a rabbit in good condition by bleeding from the heart. Using aseptic precautions immediately remove the kidneys and place each in a tube or wide-mouthed bottle of at least 37 mm. inside diameter. Holding the tube near a flame in a slanting position, strip off the capsule and cut each kidney into pieces about 5 mm. in diameter. To 16- by 133-mm. tubes transfer one piece each aseptically and shake it to the bottom. Cool the combined agar and broth to 55° and admix the ascitic fluid prewarmed to 55°. Dispense aseptically in amounts to give a depth of 35–40 mm. in the tubes containing the pieces of kidney. Cover with a 15-mm. layer of sterile mineral oil. Incubate twenty-four hours at 35°–37° and ninety-six hours at 20°–27° and inspect.

F34E. Rabbit serum semisolid agar (49) (for Leptospira)

Sodium chloride solution, 0.85-per-cent (*F64*)	200 ml.
Beef-infusion agar, 2-per-cent (*F34B*)	25 ml.
Rabbit serum, sterile	25 ml.
Laked rabbit blood, sterile	3–5 ml.

Prepare the laked blood by adding aseptically 9 ml. of sterile water to 1 ml. of sterile rabbit blood; admix 3–5 ml. with the serum and sterilize by filtration. Mix the melted agar base *F34B* and the salt solution, and autoclave twenty minutes. Cool to 55°, aseptically combine the agar and serum mixtures and dispense in tubes aseptically as required. Incubate a few tubes from each lot forty-eight hours at 35°–37° and inspect. Store the remainder at 4°–6°.

F35: A, B, C. Beef-infusion agar: A. With 0.2-per-cent glucose; B. With glucose and cystine (50); C. With glucose, cystine, and blood (51)

Beef infusion, concentrated (*F1B*)	500 grams
Agar	17.5 grams

Peptone	10	grams
Sodium chloride	5	grams
Water	500	grams
A. Glucose	2	grams per kg.
B, *C*. Cystine finely ground	0.1	gram per 100 ml.
B, *C*. Glucose solution, 20-per-cent (*F62A*)	5	ml. per 100 ml.
C. Rabbit blood, defibrinated, sterile	5–10	ml. per 100 ml.

Dissolve the agar in the water by autoclaving, the peptone and salt in the infusion, combine and make up to total weight. Adjust pH to 7.8.

A. *Filter through cotton and gauze or by aspiration,* weigh the filtrate recovered, add the glucose, and dissolve. Dispense 15-ml. amounts in 19- by 150-mm. tubes and autoclave twenty minutes.

B and *C*. Filter through cotton. Usually dispense 100-ml. amounts in 500-ml. flasks and autoclave twenty minutes. The final pH should be 7.3–7.5. Add the cystine to the agar base and heat in steam at 100° for thirty minutes. Mix thoroughly and cool to 45°. Aseptically add the glucose and in the case of *C*, the blood. Dispense as ordered. Incubate twenty-four hours at 20°–27° and inspect.

F36: A, B, C. Beef-infusion agar, semisolid: A. With ascitic fluid; B. With glucose; C. With serum (52)

	A, *B*	*C*	
Beef infusion, concentrated (*F1B*)	500	500	grams
Agar	5	22.5	grams
Sodium chloride	5	5	grams
Peptone	10	10	grams
Water	500	500	grams
A. Ascitic fluid, sterile	an equal volume		
B. Glucose	10 grams per kg.		
C. Horse serum (or rabbit serum if specified), sterile	750 ml. per 150 ml.		

Dissolve the agar in the water by autoclaving, and the peptone and salt in the infusion. Combine. Make up to total weight. Adjust pH: *A* and *C* to 7.5, *B* to 7.8. *A*, *B*, and *C*. *Filter through cotton and gauze or by aspiration.*

A. Usually dispense 400-ml. amounts in 2-liter flasks and autoclave thirty minutes. Store. Melt the agar base and cool to 50°. Warm the ascitic fluid to 50° and combine aseptically. Mix well and dispense with aseptic precautions 4-ml. amounts in 13- by 125-mm. tubes. Cover the medium aseptically with about 0.5 ml. of sterile mineral oil. Cool in an upright position. Incubate forty-eight hours at 35°–37°, and ninety-six hours at 20°–27°. Inspect and store.

B. Weigh the filtrate recovered and add the glucose. Dispense as specified in 16- by 133-mm. tubes and autoclave twenty minutes.

C. Dispense 150-ml. amounts in 2-liter flasks and autoclave thirty minutes. Store. As required, melt the agar base and cool to 50°; warm the serum to 50° and combine aseptically. Mix well and dispense aseptically 4-ml. amounts in 13- by 125-mm.

tubes. Allow to cool in an upright position. Trim plugs and seal with paraffin. Incubate half the lot at 35°–37° and the rest at 20°–27° for forty-eight hours. Inspect. Then incubate 5 days at 20°–27°, inspect again, and store at 4°–6°.

F37. Beef-infusion agar with coagulated blood (53) (for Neisseria gonorrhoeae)

Beef	450 grams
Peptone	10 grams
Disodium phosphate, Na_2HPO_4	2 grams
Water	1000 grams
Agar	13 grams per kg.
Horse blood, defibrinated, sterile	10 ml. per 100 ml.

Dissolve the peptone and phosphate in the water and add to the meat. Infuse at 55°–60° for forty-five minutes and then in steam at 100° for thirty minutes. Make up weight to 1462 grams and adjust pH to 7.4. Filter through glass wool, weigh, add the required amount of agar, and dissolve by autoclaving. Dispense as required and autoclave fifteen minutes. Store. As required, melt and admix the blood aseptically at 85°–90°. Rapidly dispense 12–20 ml. in Petri plates, or slant convenient amounts in tubes. This medium is not incubated at 35°–37° before use.

F38. Beef-infusion agar, alkaline blood (54) (for Vibrio cholerae)

Beef infusion, concentrated (*F1B*)	500 ml.
Agar	30 grams
Peptone	10 grams
Sodium chloride	5 grams
Water	500 ml.
Horse blood, defibrinated, sterile	15 ml. per 70 ml.
Normal sodium hydroxide	15 ml. per 70 ml.

Dissolve the agar in the water by autoclaving, the peptone and salt in the infusion, combine, adjust pH to 6.8, and filter through glass wool. Dispense 70 ml. amounts in 500-ml. flasks and autoclave twenty minutes. Store. Separately mix equal volumes of defibrinated horse blood and *N* NaOH and heat in steam at 100° for thirty minutes. Combine aseptically 30 ml. of this with 70 ml. of melted agar base. Dispense about 15-ml. amounts in Petri plates with porous earthenware covers. Incubate twenty-four hours at 35°–37°. Inspect.

F39: A, B. Beef-infusion ascitic fluid agar with indicator and carbohydrate: A. Two-per-cent agar; B. Five-tenths-per-cent agar (semisolid) (52, 55)

	A	*B*
Sugar-free beef infusion, concentrated (*F1C*)	500	500 grams
Agar	20	5 grams
Sodium chloride	5	5 grams
Peptone	10	10 grams
Water	500	500 grams
Phenol red, 0.02-per-cent solution	30 ml. per kg.	

Ascitic fluid, sterile	10 ml. per 100 ml.
Carbohydrate, biologically tested, as specified	

Dissolve the agar in the water by autoclaving, the salt and peptone in the infusion, combine, and make up to total weight. Adjust pH to 7.8. *Filter through cotton and gauze or by aspiration.* Weigh the filtrate; adjust pH to 7.6. Add the phenol red solution and dispense as required—usually 200-ml. amounts in 500-ml. flasks. Autoclave twenty minutes. Store. As required, melt the agar base. Cool to 50°, combine aseptically with the carbohydrate in solution (*F62*) and the ascitic fluid, both previously warmed to 50°; mix thoroughly and dispense. *A*. Dispense aseptically about 2.5-ml. amounts in 13- by 125-mm. test tubes with cotton-cork combination plugs. Slant and allow to solidify. Incubate forty-eight hours at 35°–37°, then forty-eight hours at 20°–27°. Inspect and store. *B*. Dispense aseptically about 2-ml. amounts in 11- by 75-mm. tubes. Trim the plugs and seal with paraffin. Incubate forty-eight hours at 35°–37°, then forty-eight hours at 20°–27°. Inspect and store.

F40: A, B, C. Beef-infusion serum glucose agar; B. with tellurite (56); C. With tellurite and copper sulfate (57)

	A, B	*C*
Beef infusion, concentrated (*F1B*)	500	500 grams
Agar	15	20 grams
Sodium chloride	5	5 grams
Peptone	10	10 grams
Water	500	500 grams

A, B, C.	Horse serum, sterile	50 ml. per liter
A, B, C.	Glucose, 20-per-cent solution (*F62A*)	10 ml. per liter
B, C.	Potassium tellurite, 1-per-cent solution	10 ml. per liter
C.	Copper sulfate, $CuSO_4 \cdot 5H_2O$, 10-per-cent solution	1 ml. per liter

Dissolve the agar in the water by autoclaving, and the peptone and salt in the infusion. Combine and make up to total weight.

A and *B*. Adjust pH to 7.6. *Filter through cotton and gauze or by aspiration.* Dispense 1-liter amounts in 2-liter flasks and autoclave thirty minutes. Store.

A. Melt the agar base and cool to 50°. Warm the glucose solution and serum to 50° and combine aseptically. Mix well. Dispense 15- to 20-ml. amounts aseptically in glass-covered Petri plates through a sterile drain. Incubate overnight at 35°–37° and inspect.

B. Proceed as in *A*, except to make the combination at 55° (including the tellurite) and incubate at 20°–27°.

C. Adjust pH to 8.4 and *filter through cotton and gauze or by aspiration.* Adjust pH to 8.2–8.3, and dispense 1000-ml. amounts in 2-liter flasks. Autoclave thirty minutes and store. The medium should have a pH of 8.0 after sterilization. If necessary, titrate a sample to pH 8.0. As required, melt the agar base and add *N* NaOH aseptically in an amount calculated to give the required pH. Cool to 55°, bring other ingredients to the same temperature, admix aseptically, and dispense 15- to 20-ml. amounts in glass-covered Petri plates.

NOTE: The copper sulfate solution is made up in small quantity from the U.S.P. salt ($CuSO_4 \cdot 5H_2O$) and autoclaved twelve minutes.

Potassium tellurite solution

Potassium tellurite	1 gram
Water to make	100 ml.

Grind potassium tellurite very fine in a small, dry mortar, add 10 ml. of water, and grind well again. Add 20 ml. of water, stir, allow sediment to settle, and decant supernatant fluid into a 100-ml. graduate. Repeat the process twice more. With water as needed, rinse contents of the mortar into the graduate, and make up to 100-ml. Sterilize by filtration and dispense aseptically as required.

NOTE: The potassium tellurite has been about 60-per-cent K_2TeO_3 and contained some insoluble material. A slight change may occur upon filtering the solution, but is usually negligible (56).

F43. A, B. Beef-heart-infusion agar: A. With blood; B. With glucose and ascitic fluid

Beef heart	450 grams
Water	500 grams
Agar	20 grams
Sodium chloride	5 grams
Peptone	10 grams
Water	500 grams
A. Horse, sheep, or rabbit blood, defibrinated, sterile	50 ml. per 900 ml.
B. Glucose, 20-per-cent solution (*F62A*)	34 ml. per 900 ml.
Ascitic fluid, sterile	90 ml. per 900 ml.

Infuse the meat overnight at 4°–6°, then one hour at 45°–50°, with stirring. Boil thirty minutes, strain through a colander, filter through glass wool, chill, and remove fat. Make up weight to 500 grams. Adjust pH to 7.6–7.8, boil five minutes, and make up weight. Dissolve the peptone in this infusion, the agar and salt in the remaining water by autoclaving, combine, stir thoroughly, and adjust pH to 7.4. Filter through glass wool. Dispense 900 ml. in 2-liter flasks and autoclave thirty minutes. Store.

A. Melt the agar base, cool to 45°, aseptically admix the blood (horse blood unless otherwise specified), attach a sterile drain, and dispense as required. For slants, use about 3-ml. amounts in 13- by 125-mm. tubes, incubate twenty-four hours at 35°–37°, inspect, and store at 20°–27°. For plates, use 15- to 20-ml. amounts in glass-covered Petri plates. Incubate twenty-four hours at 35°–37°, inspect, and store at 20°–27°.

B. Melt the agar base and cool to 50°. Aseptically add the other ingredients in the given order and mix thoroughly. Attach a sterile drain, dispense aseptically, and slant: 65-ml. amounts in 500-ml. flatsided bottles, or 5-ml. amounts in 19- by 150-mm. tubes. Incubate forty-eight hours at 35°–37°. Inspect, store at 20°–27° for five days, and inspect again.

F43.5. Beef-infusion agar with glucose and blood (for Trypanosoma) (58)

Beef infusion, concentrated (*F1B*)	250	grams
Water	750	grams
Peptone	25	grams
Sodium chloride	7	grams
Agar (Bacto)	10	grams

Glucose solution, 20-per-cent (*F62A*)	0.5	ml. per 100 ml.
Rabbit blood, defibrinated, sterile	10	ml. per 100 ml.

Combine the infusion and water, add the peptone and salt, and dissolve. Filter through cotton. Adjust pH to 6.8–7.0. Add the agar and dissolve by autoclaving. Dispense in 100-ml. amounts in 300-ml. flasks and autoclave at 115°–118° for thirty minutes. As required melt the agar base, cool to 50°–55°. Admix the glucose and blood and dispense aseptically 5-ml. amounts in 16- by 133-mm. tubes. Slant the tubes to give a short slanted surface. Promptly after the medium has hardened, paraffin the cotton plugs and store the tubes at 4°–6° twelve hours to promote condensation on the slanted surface. Incubate at 35°–37° twelve to twenty-four hours. Inspect.

* NOTE: Beef extract (Bacto), 50 grams dissolved in 1000 grams of water may be used instead of the meat infusion, and guinea pig instead of rabbit blood.

F44. Meat-infusion desoxycholate citrate agar (59)

Horse meat, beef, or pork	1000	grams
Water	3000	grams
Normal hydrochloric acid	10	ml.
Normal sodium hydroxide	10	ml.
Peptone (Wilson's thio)	30	grams

Agar (Merck), U.S.P.	20	grams per kg.
Normal sodium hydroxide	5	ml. per kg.
Lactose	10	grams per kg.
Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$	20.6	grams per kg.
Sodium desoxycholate, (Riedel-deHaen)	5	grams per kg.
Lead chloride, $PbCl_2$, 0.35-per-cent solution	1	ml. per kg.

Ferric ammonium citrate, green scales (Merck)	2	grams per liter
Neutral red, 1-per-cent solution	2	ml. per liter

Infuse the meat at room temperature for one hour. Add the HCl with stirring. Boil thoroughly one minute, strain, and remove fat. Make up to 3000 grams. Add the NaOH and the peptone and adjust pH to 8.0. Boil thoroughly three minutes and make up weight. Filter through paper and weigh the filtrate. Per kilogram recovered incorporate the other ingredients, in the proportions given, as follows:

Add the agar and NaOH. Stir and allow to stand fifteen minutes. Heat in steam at 100° for thirty minutes. Admix the lactose, citrate, desoxycholate, and lead chloride in the order given. Dispense 500-ml. amounts in 1-liter flasks or 1000-ml. amounts in 2-liter flasks, and store.

As required melt the agar base and admix the ferric ammonium citrate and the neutral red solution. Adjust pH to 8.0, using phenol red as indicator in the titration. Dispense about 15-ml. amounts in Petri plates with porous earthenware covers.

F45: A, B. Veal-infusion agar, semisolid (60, 11) (for Clostridium tetani)

	A	*B*
Veal infusion, concentrated (*F1B*)	500	500 grams
Sodium chloride	5	5 grams
Peptone (Berna)	10	0 grams
Peptone (Difco neopeptone)	0	10 grams
Agar	5	3 grams
Water	500	500 grams

Dissolve the agar in the water by autoclaving; the peptone and salt in the infusion; combine, make up weight, and adjust pH to 7.8. *Filter through cotton and gauze or by aspiration.* Dispense about 15-ml. amounts in 19- by 150-mm. tubes and autoclave twenty minutes.

F46. Veal-infusion agar with glucose

Veal infusion, concentrated (*F1B*)	500	grams
Agar	20	grams
Sodium chloride	5	grams
Peptone	10	grams
Water	500	grams
Glucose	7.5	grams per kg.

Dissolve the agar in the water by autoclaving; the peptone and salt in the infusion; combine and make up weight. Adjust pH to 7.6–7.8, filter through cotton and weigh filtrate. Add the glucose and stir to dissolve. Dispense 65-ml. amounts in 500-ml. flat-sided bottles, autoclave thirty minutes, and slant.

F47: A, B. Veal-infusion glycerol agar (for Hemophilus influenzae): A. With coagulated blood (61); B. With blood extract

Veal-residue infusion (*F1D*)	1000 grams
Agar	25 grams
Sodium chloride	5 grams
Peptone	10 grams
Glycerol	40 ml. per kg.
A. Horse or sheep blood, defibrinated, sterile	5 ml. per 100 ml.
B. Blood extract (*F58*)	10 ml. per 100 ml.

Dissolve the agar in half the infusion by autoclaving, and the salt and peptone in the remainder. Combine and make up to weight. Adjust pH to 8.2, filter through cotton, weigh the filtrate, and admix the glycerol. Dispense 200-ml. amounts in 500-ml. flasks and autoclave twenty minutes. Store. Melt the agar base and hold at 90°–100° till used. Aseptically admix (*A*) the blood, or (*B*) the blood extract, where a relatively clear medium is desired; and dispense as required. Incubate twenty-four hours at 35°–37°, inspect, and store.

F48. Beef-extract agar, alkaline egg (62, 63) (for Vibrio cholerae)

Agar base	90 ml.
Alkaline egg mixture	25 ml.

Admix the egg preparation aseptically with the melted agar base and dispense 15- to 20-ml. amounts in Petri plates with porous earthenware covers. Incubate twenty-four hours at 35°–37°, and inspect.

Agar base

Agar (Bacto)	30 grams
Peptone	10 grams
Sodium chloride	5 grams
Beef extract	3 grams
Water	1000 grams
Glucose	5 grams per kg.

Dissolve the agar in nine-tenths of the water by autoclaving, and the other ingredients in the remainder. Combine and make up weight. Filter through cotton, weigh, and add the glucose. Dispense 90-ml. amounts in 100-ml. French square bottles and autoclave twenty minutes.

Alkaline egg mixture

Egg contents	50 ml.
Water	50 ml.
Sodium carbonate, Na_2CO_3	6.5 grams
Water to make	100 ml.

Mix the egg contents and water in a beaded flask and add an equal volume of 6.5-per-cent sodium carbonate. Mix thoroughly and filter through glass wool. Dispense 30-ml. amounts in 50-ml. flasks or in 25- by 175-mm. tubes and heat in steam at 100° for thirty minutes.

F48.5. Beef-extract agar, semisolid (64) (for Bacterium typhosum)

Agar	3 grams
Sodium chloride	5 grams
Beef extract	3 grams
Peptone	10 grams
Water	1000 grams

Dissolve the agar in half the water by autoclaving, and the salt, extract, and peptone in the remainder. Combine, make up to total weight, and adjust pH to 7.2. Filter through cotton or cotton and gauze. Dispense 4-ml. amounts in 16- by 133-mm. test tubes and insert in the center of each a 65-mm. length of glass tubing, outside diameter 8 mm., wall thickness 1 mm., open at both ends. Autoclave twenty minutes.

F49A. Beef-extract agar, 1.5-per-cent (13d) (for bacteriologic examination of water)

Agar	15 grams
Beef extract	3 grams
Peptone	5 grams
Water	1000 grams

Dissolve the agar in half the water by autoclaving, and the beef extract and peptone in the remainder; combine, and make up weight. Adjust pH to 6.6, filter through cotton or cotton and gauze, dispense about 15-ml. amounts in 19- by 150-mm. tubes, and autoclave twenty minutes. The final pH should be 6.4–7.0.

F49: B, C. Beef-extract tryptone glucose agar, 1.5-per-cent: B. With skim milk (65a) (for bacteriologic examination of milk); C. Plain (66, 67) (for bacteriologic examination of water)

	B, C
Agar (purified)	15 grams
Beef extract (Bacto)	3 grams
Tryptone	5 grams
Glucose	1 gram
Water	1000 grams
B. Skim milk (*F18A* before heating) or reconstituted spray process skim milk	10 ml. per 1024 grams

B, C. Dissolve the agar in half the water by autoclaving and the beef extract, tryptone, and glucose in the remainder; combine and make up weight. Adjust pH to 7.0 and filter through cotton and gauze. *B*. Cool to 48°–50° and weigh. Add the fluid milk or spray process skim milk powder previously dispersed in water (10 grams powder per 100 ml. water) and dispense rapidly about 15-ml. amounts in 19- by 150-mm. tubes. Autoclave at 115°–118° for twenty minutes. *C*. Dispense about 15-ml. amounts in 19- by 150-mm. tubes. Autoclave at 115°–118° for twenty minutes.

Special precautions must be taken in the preparation of the medium containing milk to avoid the development of a precipitate during sterilization. Some brands of commercial agar or even some lots of the same brand may contain impurities that make it unsatisfactory for use without further purification, such as washing (68). A precipitate may also develop if the period of heating is prolonged; accordingly, the total time in the autoclave should not exceed one hour.

F49.5. A, B. Beef-extract agar: A. pH 6.0; B. With glycerol pH 7.8 (69) (for bacteriophage typing of Bacterium typhosum)

	A	*B*
Agar	15	15 grams
Sodium chloride	5	5 grams
Beef extract	3	3 grams
Peptone	10	10 grams
Glycerol	0	10 ml.
Water	1000	1000 grams

Dissolve the agar in half the water by autoclaving and the other ingredients in the remainder. Combine; make up to total weight and adjust pH: *A* to 6.0–6.2, *B* to 7.8–7.9. Filter through cotton or cotton and gauze and dispense as specified. *A*. Dispense 2- to 3-ml. amounts in 13- by 125-mm. test tubes. Autoclave twenty minutes. Slant. *B*. Dispense in convenient amounts and autoclave twenty minutes. Store. As required, melt and redispense 15- to 20-ml. amounts in Petri plates.

F50: A. B. Beef-extract agar with indicator: A. With lactose; B. With lactose and glucose (70, 71)

	Agar	15	grams
	Sodium chloride	5	grams
	Beef extract	3	grams
	Peptone	10	grams
	Water	1000	grams
	Andrade's indicator	30	ml. per kg.
	Lactose (biologically tested)	10	grams per kg.
B.	Glucose	0.5	gram per kg.

Dissolve the agar in half the water by autoclaving and the peptone, extract, and salt in the remainder; combine, make up weight, and filter through cotton. Admix the indicator and adjust pH to 7.4–7.6. Add the lactose (in the case of *B* add the glucose also), and stir to dissolve. Dispense in 13- by 125-mm. tubes about half full, autoclave twelve minutes, and store. When required, melt, and slant with a deep butt.

Andrade's indicator (72)

Acid fuchsin (Coleman and Bell)	0.1–0.5	gram
Normal sodium hydroxide	16	ml.
Water	100	ml.

The amount of dye required is determined by standardization tests made with each new lot of fuchsin, see p. 310. Dissolve the required amount of fuchsin in the water, add the alkali, and mix thoroughly. Let stand from one to two hours, filter through paper, and dispense as required in brown glass bottles.

F50.5. Bile salts citrate agar (73a)

Agar	17	grams
Beef extract	5	grams
Peptone (Difco proteose)	5	grams
Bile salts, No. 3 (Difco)	8.5	grams
Water	1000	grams
Lactose	10	grams per liter
Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$ (A.C.S.)	8.5	grams per liter
Sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$ (A.C.S.)	8.5	grams per liter
Ferric citrate pearls, U.S.P. VIII, 10-per-cent solution	10	ml. per liter
Neutral red (Coleman and Bell), 1-per-cent solution	2.5	ml. per liter
Brilliant green (Coleman and Bell), 0.1-per-cent solution	0.33	ml. per liter

Dissolve the agar in half the water by autoclaving, and the extract, peptone, and bile salts in the remainder; combine and make up weight. Dispense as ordered, autoclave thirty minutes, and store.

As required, melt a suitable amount of agar base, add in the proportions given the lactose, sodium citrate, sodium thiosulfate, and ferric citrate solution and mix thoroughly. Adjust pH to 7.0. Admix the neutral red and brilliant green solutions and dispense 15- to 20-ml. amounts in Petri plates with porous earthenware covers.

F51. Bismuth sulfite agar (74–76)

Agar base

Beef extract (Bacto)	5 grams
Peptone	10 grams
Water, hot, to make	1000 ml.
Agar (Bacto or Reclaimed *F59.5*)	20 grams

To the agar in a 2-liter flask add a hot prepared solution of the other ingredients. Dissolve and sterilize by autoclaving fifteen minutes. Remove from sterilizer and mix thoroughly. Use at once or store.

Variable results (77) obtained with different brands of agar and different lots of the same brand have emphasized the importance of this ingredient and the need for using a highly purified product.

Bismuth sulfite mixture

1. Bismuth ammonia citrate, U.S.P.	6 grams
Water, boiling	50 ml.
2. Sodium sulfite, Na_2SO_3	10–20 grams
Water, boiling	100 ml.
3. Glucose	10 grams
Water, boiling	50 ml.
4. Disodium phosphate, Na_2HPO_4	10 grams

For solution 1, the U.S.P. IX "Bismuth and ammonium citrate," sold in this country under the name of bismuth ammonium citrate may be used. The proportion of sulfite in solution 2 may be varied within the given limits as a means of standardizing the medium with different lots of ingredients, e.g., agar or peptone.

Make solutions 1, 2, and 3 separately. Mix 1 and 2, boil, and admix 4 slowly and carefully while boiling gently. Allow to cool and admix 3. Make up weight. Store in dark cupboard at 20°–27°.

Iron citrate, brilliant green solution

Iron citrate, U.S.P. (ferric citrate)	1 gram
Water, boiling	100 ml.
Brilliant green (Grübler), 1-per-cent solution	12.5 ml.

Dissolve the citrate in the water and admix the brilliant green. Store as above.

Combined medium

Agar base	1000 ml.
Bismuth sulfite mixture	200 ml.
Iron citrate, brilliant green solution	45 ml.

With the melted agar at 55°–100° admix the other ingredients and pour immediately 15- to 20-ml. amounts in Petri plates with porous earthenware covers. Store at 4°–6° after one to two hours.

F52A. Endo agar (78, 79) (for bacteriologic examination of feces)

Agar	25 grams
Peptone	10 grams
Beef extract	5 grams
Water	1000 grams
Sodium carbonate, Na_2CO_3, 10-per-cent solution	10 ml. per kg.
Lactose	10 grams per kg.
Basic fuchsin, saturated alcoholic solution	a standardized amount
Sodium bisulfite, $NaHSO_3$ (A.C.S.), 10-per-cent solution	10 ml. per kg.

Dissolve the agar in half of the water by autoclaving, and the extract and peptone in the remainder; combine and make up weight. Filter through cotton. Add the sodium carbonate, mix, and determine pH, usually 7.6–8.0. Heat in steam at 100° for ten minutes, add the lactose, and an amount of fuchsin determined by standardization of the lot of dye used, see p. 310. Stir to dissolve. Admix the sodium bisulfite which should cause a previously red color to fade. Dispense 750-ml. amounts in 1-liter bottles and autoclave at 115°–118° for twenty minutes.

Basic fuchsin solution, saturated alcoholic

Fuchsin, basic	10 grams
Alcohol, 95-per-cent, to make	100 ml.

Add the fuchsin to part of the alcohol in a 100-ml. glass-stoppered volumetric flask. Shake well and make up to volume. Let stand overnight, filter through paper, and dispense in glass-stoppered bottles.

Sodium bisulfite solution, 10-per-cent

Sodium bisulfite, $NaHSO_3$ (A.C.S.)	10 grams
Water to make	100 ml.

Dissolve the bisulfite in part of the water in a 100-ml. volumetric flask and make up to volume. Filter through paper. Keep under a 2-cm. layer of mineral oil in a bottle with a siphon.

F52B. Endo agar (13d, 78, 80) (for bacteriologic examination of water)

Agar	30	grams
Beef extract	5	grams
Peptone	10	grams
Lactose	10	grams

Water	1000	grams
Basic fuchsin, 3-per-cent alcoholic solution	6	ml. per 600 ml.
Sodium sulfite, anhydrous (dissolved in 30 ml. of distilled water)	0.75	grams per 600 ml.

Dissolve the agar in nine-tenths of the water by autoclaving, and the extract, lactose, and peptone in the remainder; combine and make up to weight. Adjust pH to 7.8 (pH after sterilization should be 7.4). Filter through cotton or cotton and gauze. Dispense 600-ml. amounts in 1-liter flasks and autoclave thirty minutes. Store. As required, melt the agar, admix the fuchsin solution and the freshly prepared sulfite solution and dispense 20- to 25-ml. amounts in Petri plates with porous earthenware covers.

Basic fuchsin solution

Basic fuchsin, certified	3 grams
Alcohol, 95-per-cent, to make	100 ml.

Dissolve the fuchsin in part of the alcohol and make up to volume. Allow to stand twenty-four hours. Filter through paper and store.

F52.4. Eosin methylene blue agar (81) (for bacteriologic examination of feces)

Agar	20	grams
Sodium chloride	5	grams
Beef extract	3	grams
Peptone	10	grams
Water	1000	grams
Lactose, 10-per-cent solution (*F62A*)	37.5	ml. per 750 ml.
Eosin, 3-per-cent solution	10	ml. per 750 ml.
Methylene blue, 0.5-per-cent solution	15	ml. per 750 ml.

Dissolve the agar in half of the water by autoclaving, and the extract and peptone in the remainder; combine and make up weight. Adjust pH to 7.0–7.2 and filter through cotton. Dispense 750-ml. amounts in 1-liter bottles and autoclave thirty minutes. Store. Melt the agar base. Admix the other ingredients in the order given and dispense 20- to 25-ml. amounts in Petri plates with porous earthenware covers.

Eosin solution, 2- or 3-per-cent

Eosin, yellowish (National Aniline)	20 or 30 grams as specified
Water to make	1000 ml.

Dissolve the dye, make up to volume, let stand overnight, filter through paper, and dispense in glass-stoppered bottles.

Methylene blue solution, 0.5-per-cent

Methylene blue (National Aniline)	5 grams
Water to make	1000 ml.

Dissolve the dye in part of the water and make up to volume. Let stand overnight, filter through paper, and dispense in glass-stoppered bottles.

F53. Eosin methylene blue agar (13e) (for bacteriologic examination of water)

Peptone	10 grams
Dipotassium phosphate, K_2HPO_4	2 grams
Agar	15 grams
Water	1000 grams
Lactose, 20-per-cent solution (*F62A*)	5 ml. per 100 ml.
Eosin, yellowish, 2-per-cent solution	2 ml. per 100 ml.
Methylene blue, 0.5-per-cent solution	2 ml. per 100 ml.

Combine the ingredients above the line, boil till dissolved, make up weight, dispense as required, and autoclave fifteen minutes. Store. As needed, melt a suitable amount of base, add the other ingredients in the indicated proportion, mix thoroughly, and dispense 20- to 25-ml. amounts in Petri plates with porous earthenware covers. See *F52.4* for preparation of dye solutions.

F54. Asparagine serum agar (82) (for Endamoeba histolytica)

Ringer's solution (*F63.1*)	500 ml.
Agar (Bacto)	7.5 grams
Asparagine	0.5 gram
Horse serum, sterile	25 ml.
Ringer's solution (*F63.1*)	500 ml.

Dissolve the agar in the first portion of Ringer's solution by autoclaving, make up weight, and admix the asparagine. Dispense 6-ml. amounts in 13- by 125-mm. tubes, autoclave twenty minutes, and slant to give a 2-cm. butt and slant-length about 5 cm. Leave at room temperature overnight. Autoclave the second portion of Ringer's solution in a 2-liter flask for twenty minutes. The next day heat the required amount of horse serum thirty minutes at 55°, admix aseptically with the Ringer's solution, and add aseptically enough of the mixture to each of the slants to rise 1 cm. above the solid medium with the tube upright. Incubate forty-eight hours at 35°–37° and inspect.

F54.5. Blood agar (83, 84) (for Leishmania)

Agar (Bacto)	15.5 grams
Sodium chloride	6.5 grams
Water	1000 grams
Rabbit (or guinea pig) blood, defibrinated, sterile	10 ml. per 150 ml.

Dissolve the agar and salt in the water by autoclaving. Make up weight. Adjust pH to 6.8–7.0. Dispense 150-ml. amounts in 300-ml. flasks. Autoclave at 115°–118° for thirty minutes. As required, melt the agar base, cool to 50°–55°, admix the blood and dispense aseptically 5-ml. amounts in 16- by 133-mm. tubes. Slant to give a long slanted surface. As soon as the medium has hardened, paraffin the cotton plugs and place the tubes in the cold room at 4°–6° for twelve hours, then incubate at 35°–37° for twelve to twenty-four hours. Inspect.

F55. Citrate agar (85)

Agar (Bacto)	20	grams
Sodium chloride	5	grams
Magnesium sulfate, $MgSO_4$	0.2	gram
Monoammonium phosphate, $NH_4H_2PO_4$	1	gram
Dipotassium phosphate, K_2HPO_4	1	gram
Sodium citrate		
$Na_3C_6H_5O_7$	2	grams
or $Na_3C_6H_5O_7 \cdot 2H_2O$	2.28	grams
or $2Na_3C_6H_5O_7 \cdot 11H_2O$	2.77	grams
Water	1000	grams
Bromothymol blue (LaMotte), 0.4-per-cent solution	20	ml. per kg.

Dissolve the agar in half of the water by autoclaving and the salts in the remainder; combine and make up weight. Adjust pH to 6.8. Dispense as required. Autoclave twenty minutes. As required melt the agar base. Aseptically admix the bromothymol blue indicator, and dispense 3- to 4-ml. amounts in 13- by 125-mm. tubes. Slant and incubate at 35°–37° for twenty-four hours. Inspect.

NOTE: The indicator solution is prepared by grinding 0.1 gram of powdered bromothymol blue (LaMotte) in an agate mortar with 3.2 ml. of 0.05 *N* NaOH until dissolved and then diluting to 25 ml. with water.

F55.2. Serum agar, infusion-free, with glucose (for Neisseria meningitidis)

Agar	20 grams
Sodium chloride	5 grams
Dipotassium phosphate, K_2HPO_4	1 gram
Monopotassium phosphate, KH_2PO_4	1 gram
Peptone	10 grams
Water	1000 grams
Horse serum, sterile	100 ml. per liter
Glucose, 20-per-cent solution (*F62A*)	50 ml. per liter

Dissolve the sodium chloride and agar in half the water by autoclaving; the phosphates and peptone in the remainder by heat. Combine and make up to total weight. Adjust pH to 7.4–7.6. Filter through cotton or cotton and gauze. Dispense 1000-ml. amounts in 2-liter flasks. Autoclave thirty minutes and store. As required, melt the agar base and cool to 50°. Warm the serum and glucose solution to 50°, combine aseptically with the melted agar. Mix well and dispense aseptically 7-ml. amounts in 19- by 150-mm. tubes. Slant, allow to solidify, incubate forty-eight hours at 35°–37°, then twenty-four hours at 20°–27°. Inspect and store.

F55.5: A, B. Glucose agar, infusion-free, with coagulated blood (73b) (for Neisseria gonorrhoeae and Neisseria meningitidis): B. With gelatin (86) (for N. gonorrhoeae culture outfits)

	A	*B*	
Agar	15	20	grams
Gelatin	0	40	grams
Sodium chloride	5	5	grams

	A	*B*	
Disodium phosphate, Na_2HPO_4	5	5	grams
Peptone (Difco proteose No. 3)	20	20	grams
Glucose	0.5	0.5	gram
Water to make	1000	1000	grams
Nile blue A, 0.04-per-cent solution, sterile	0	0.6	ml. per 80 ml.
Horse blood, defibrinated, sterile	10 ml. per 100 ml.	20	ml. per 80 ml.

Dissolve the agar in half the water by autoclaving; the salts, peptone, and glucose, (*B* and gelatin) in the remainder with heat. Combine, make up weight to 1 kg. and adjust pH to 7.4–7.6. Dispense as specified and autoclave twenty minutes. Store.

As required, melt the agar base. Admix the blood or the blood and Nile-blue-A solution aseptically at 85°–90°. Rapidly dispense the medium aseptically. *A*. Dispense 15- to 20-ml. amounts in glass-covered Petri plates. Deliver without incubation. *B*. Dispense 6-ml. amounts in sterile 16- by 133-mm. test tubes stoppered with paraffined corks. The mixture is thick and difficult to handle and hardens quickly.

NOTE: The tubes used for *B* should have corks that are the same size as those in the swab tubes prepared for the culture outfits.

F55.7. Starch gelatin agar, infusion-free, with indicator and carbohydrate (114) (for Neisseria gonorrhoeae and Neisseria meningitidis)

Agar	3 grams
Gelatin	10 grams
Sodium chloride	5 grams
Peptone (Difco proteose No. 3)	10 grams
Starch, soluble, powdered	5 grams
Water to make	1000 grams
Phenol red, 0.02 per cent	30 ml. per kg.
Carbohydrate, biologically tested	10 grams per kg.

Dissolve the agar in half the water by autoclaving; the gelatin, salt, peptone, and starch in the remainder with heat. Combine and make up to total weight. Adjust pH to 7.4–7.6. Filter through cotton. Weigh the filtrate recovered and add the indicator and carbohydrate. Dispense 2.5-ml. amounts in 11- by 75-mm. tubes and autoclave at 115° for twelve minutes. Trim the plugs and seal with paraffin.

F56: A, B. Potato-infusion blood agar (87–88): B. With peptone (89) (for Hemophilus pertussis)

	A	*B*
Potato infusion	250	250 grams
Peptone (Difco proteose)	0	10 grams
Agar (Bacto)	25	25 grams
Sodium chloride	5	5 grams
Water	750	750 grams
Sheep or rabbit blood, defibrinated, sterile	90 ml. per 500 ml.	90 ml. per 500 ml.

Dissolve the salt and agar in the water by autoclaving. Check and make up weight. Add the potato infusion (*B* and peptone). Mix thoroughly, dispense as required, and autoclave twenty to thirty minutes. Store. Melt the agar base as needed, cool to 45°–50°, combine aseptically with the blood, and dispense as ordered.

Potato infusion

Potatoes, peeled and ground	500 grams
Glycerol	40 ml.
Water	1000 grams

Peel the potatoes, pass through a coarse grinder, and weigh. Mix thoroughly with the water and glycerol, shake well, heat at 100° for sixty minutes, and make up weight. Strain and squeeze gently. Allow to stand and decant clear supernatant.

F56.2. Starch peptone agar with citrated blood (for Hemophilus pertussis vaccine preparation)

Disodium phosphate, Na_2HPO_4	1.5	grams
Peptone (Difco proteose No. 3)	10	grams
Starch, soluble, powdered	2	grams
Glycerol	10	ml.
Sodium chloride	5	grams
Agar (Bacto)	25	grams
Water	1000	grams
Sheep blood, citrated, sterile (1 part 4-per-cent sodium citrate and 4 parts sheep blood as specified)	200–250	ml. per liter

Dissolve the peptone, phosphate, starch, and glycerol in half the water by boiling. Make up weight to 500 grams. Dissolve the agar and salt in the remaining 500 grams of water by autoclaving. Check weight and make up to 500 grams. Combine the peptone solution and agar, mix thoroughly, and adjust pH to 7.0. Dispense as required, usually in 4- to 6-liter amounts in 8-liter Pyrex bottles and autoclave eighty minutes. Cool to 48°–50°. Combine the blood, previously warmed to 35°–37° with the agar, mix thoroughly, and redispense aseptically approximately 140-ml. amounts in 1-liter Roux Pyrex bottles. Incubate forty-eight to seventy-two hours. Inspect.

F56.8. Potato-infusion agar with glucose (65c)

Potato infusion	1000 grams
Agar	20 grams
Glucose, technical	20 grams

Dissolve the agar and the glucose in the infusion by autoclaving at 115°–118°. Check and make up weight. Filter through cotton or cotton and gauze. Dispense as required, usually 150-ml. or 300-ml. amounts and autoclave at 115°–118° for twenty minutes. Determine the amount of 10.0-per-cent tartaric acid required to adjust 100 ml. to pH 3.5, using a 1:20 dilution of the 10.0-per-cent tartaric acid and bromophenol blue as indicator in the titration. Record amount on tags and on worker's record card. Store, melt the agar base as needed, adjust pH to 3.5 by adding aseptically the required amount of sterile 10.0-per-cent tartaric acid as indicated by previous titration. Dispense as specified.

NOTE: This medium may also be used at a pH about 5.6, and at this pH may not require any adjustment

Potato infusion

Potatoes, peeled and ground	200 grams
Water	1000 grams

Peel the potatoes, pass through a coarse grinder, and weigh. Mix thoroughly with the water and heat at 100° for sixty minutes. Make up weight. Strain.

F57.1: A, B, C. Peptone agar Sabouraud's modified (23, 90–92): A. Conservation (for preservation of cultures); B. With glucose; C. With honey

	A	*B*	*C*
Agar	18	20	20 grams
Peptone	10	10	10 grams
Glucose, technical	0	40	0 grams
Honey	0	0	60 grams
Water to make	1000	1000	1000 grams

A, *B*, and *C*. Dissolve the agar in four-fifths of the water and the peptone (*B* and glucose, *C* and honey) in the remaining water. Combine the solutions and filter through cotton or cotton and gauze. *A*. Dispense in 7-ml. amounts in 19- by 150-mm. tubes. Autoclave fifteen minutes, slant. *B* and *C*. Dispense as required, usually in 150-ml. amounts and autoclave fifteen minutes. As required, melt the agar, adjust pH to 5.6–5.8 by adding aseptically the required amount of sterile 10.0-per-cent tartaric acid as indicated by previous titration (as in *F56.8* except that methyl red is used as the indicator). Cool to 50° and dispense as specified.

[1] For medium *C*, a light-colored medium may be obtained by using a light-colored honey, such as a pure white clover honey.

F57.2. Corn-meal agar (93)

Agar	17.5 grams
Corn meal, yellow, ground	40 grams
Water	1000 grams

Dissolve the agar in one-half the water by autoclaving. Add the corn meal to the remainder of the water and heat at approximately 65° for one hour. Filter through paper. Admix the dissolved agar and the corn-meal filtrate (300–400 ml.) and filter through cotton. Dispense as required and autoclave twenty minutes. No pH adjustment is required.

F57.3. Wort agar (73c)

Agar	20 grams
Ammonium chloride, NH_4Cl	1 gram
Peptone	0.78 gram
Maltose, technical	12.75 grams
Malt extract (Difco)	15 grams
Dextrin (Difco)	2.75 grams
Glycerol	2.35 grams
Dipotassium phosphate, K_2HPO_4	1 gram
Water	1000 grams

Dissolve agar in one-half of the water by autoclaving. Dissolve other ingredients in the remaining water. Combine. Filter through cotton or cotton and gauze. Dispense as required, usually in 150- or 300-ml. amounts and autoclave fifteen minutes. As required, melt the agar, adjust pH to 4.8–5.0 by adding aseptically the required amount of sterile 10.0-per-cent tartaric acid as indicated by previous titration (as in *F56.8* except that methyl red is used as the indicator). Dispense as specified.

III. OTHER PREPARATIONS

F58. Blood extract (94)

Blood, defibrinated, sterile, usually sheep or horse	100 ml.
Sodium chloride, 0.85-per-cent solution (*F64*)	400 ml.

Mix the ingredients thoroughly in a flask, heat in a water bath and hold at 80° for fifteen minutes, shaking frequently. Filter through paper and dispense in centrifuge tubes or bottles. Centrifuge fifteen to twenty minutes, pipette off supernatant fluid, and sterilize by filtration.

F59. Ox bile (95)

Obtain bile from a recently killed ox, heat in steam at 100° for twenty minutes, and filter through paper. Dispense as required and sterilize fractionally for three 20-minute periods.

F59.5. Reclaimed agar (96)

Agar may be reclaimed from used culture media as a conservation measure or if a highly purified product is desired (see *F51*). Media satisfactory for reclaiming are those that were dispensed originally in relatively large amounts, 15 to 200 ml., and that do not contain starch, lead salts, dyes, or other inhibiting or differential agents that might be difficult to eliminate. The procedure is as follows:

Collect the used media in special containers. (Tubes and bottles may be placed upright in wire or metal baskets or enamel containers; Petri plates, with covers discarded separately, should be inverted in enamel containers.) Autoclave thirty minutes to sterilize and melt the agar. Pool and filter the hot agar through cotton and gauze. Allow to solidify, slice into small pieces, approximately 1 by 1 by 1/4 inches, and store in an enamel container at 4°–6° until from 12–15 liters have been accumulated; place in a freezing room maintained at from −5° to −7° or lower for four or five days or until frozen into a solid block. Transfer the block to an enamel container with a capacity of 30 liters or more. Tie a gauze cover over the container and through the gauze insert a glass or resistant metal tube long enough to reach to the bottom of the container and connected to a faucet by rubber tubing. Wash with running cold tap water for eight hours on each of three successive days. The floating block facilitates the washing process by keeping the pieces suspended in the water. As thawing proceeds the pieces separate and settle to the bottom. When the agar is thoroughly washed, drain off the water, determine the weight of the washed agar, and add an equal weight of distilled water. Heat in steam at 98°–100° for thirty minutes to dissolve the agar. Admix approximately 1 gram of powdered charcoal (Norite A) per kilogram, hold at 90°–100° for twenty to thirty minutes, then filter through a well-packed layer of hot paper pulp.

Place a piece of No. 3 Whatman filter paper in a Büchner funnel, pour on this about 500 ml. of hot paper pulp (*F82*) to make a layer about 3–4 mm. in thickness when packed. Apply vacuum and allow practically all of the water to drain from the pulp.

Allow the agar to solidify, then hold at −5° to −7° for four or five days or until frozen into a solid block. Remove the frozen block from the container to a gauze-covered rack placed over another container to collect the water. Leave undisturbed at room temperature to thaw and drain, spread out thinly on gauze, and dry in the air at room temperature or in a dehydrating unit at from 60°–80°. The dried product should have a moisture content of from 12–14 per cent.

F60. Protone solution (97)

Sodium chloride	8.5 grams
Water to make	1000 ml.
Protone (Bacto)	0.5 gram per liter

Dissolve the salt and make up to volume. A check by titration may be made. Admix the protone and adjust pH to 8.0–8.4. Filter through hard paper until clear. Dispense 2-liter amounts in 3-liter flasks and autoclave thirty minutes.

F60.3. Peptone solution (for dilution of toxins)

Peptone (Difco proteose)	10 grams
Water	1000 grams

Dissolve the peptone in the water. Filter through paper. Dispense as specified. Autoclave fifteen minutes.

F60.5. Peptone solution with formalin (98, 99)

Sodium chloride	5 grams
Peptone	10 grams
Water to make	1000 grams
Formalin, U.S.P.	1.43 ml. per 100 ml.

Dissolve the salt and peptone and make up weight to 1 kg. Adjust pH to 5.8. Dispense 100-ml. amounts in 200-ml. French square bottles. Autoclave fifteen minutes; pH should be 5.8–6.0. Add the formalin aseptically just before using.

F61: A, B. Calcium chloride solution, Q per cent: B. In 0.002 N HCl

Calcium chloride, $CaCl_2$ (A.C.S.)	2 Q grams
Water, distilled, freshly boiled	200 ml.

Since calcium chloride is hygroscopic, temporarily make a solution using only a part of the water so as to obtain at least the required concentration. Dilute one part of the solution to Q parts with water (or add $Q-1$ parts of water), and then use the conventional method to titrate 10 ml. of this solution with 0.1 N $AgNO_3$. The required titer is 18. Adjust the original solution if necessary as follows:

$$\frac{\textit{present titer}}{\textit{required titer}} \times \textit{present volume of solution} = \textit{required total volume.}$$

A. Dilute with water to that volume. Store in a well-stoppered bottle.

B. Add *N* HCl in amount equal to 1/500 of the required total volume and then make up to volume with water.

NOTE: The solution *A* without added HCl should be discarded if it becomes cloudy; *B* should remain clear and may be substituted for *A* wherever the small amount of HCl included is not objectionable.

F62: A, B. Carbohydrate solution: A. Heat sterilized; B. Sterilized by filtration

Carbohydrate, as specified	usually 10 or 20 grams
Water to make	100 ml.

Dissolve the carbohydrate (usually C.P. or biologically tested) and make up to volume. *A*. Filter through paper, dispense, and autoclave fifteen minutes. *B*. Sterilize by filtration.

NOTE: Where the carbohydrate solution is to serve merely as enrichment and is to be added aseptically to a medium sterilized separately, solutions sterilized by heat (procedure *A*) are usually employed. Where the carbohydrate solution is intended as a test substrate of microbic action (as fermentation tests) solutions of the relatively stable monoses (such as glucose, fructose, and galactose) and also the alcohols (such as glycerol and mannitol) are sterilized by heat (procedure *A*) whereas the relatively less stable polyoses (such as lactose, maltose, sucrose, and raffinose) are usually sterilized by filtration (procedure *B*).

F63. Locke's solution (100)

Sodium chloride	9	grams
Calcium chloride, $CaCl_2$, 1-per-cent solution (*F61*)	20	ml.
Potassium chloride	0.4	gram
Sodium bicarbonate, $NaHCO_3$	0.2	gram
Glucose	2.5	grams
Water	1000	ml.

Dissolve the ingredients with stirring and make up to volume. Use immediately or sterilize by filtration and store.

F63.1. Ringer's solution (101)

Sodium chloride	6	grams
Potassium chloride	0.075	gram
Calcium chloride, $CaCl_2$, 1-per-cent solution (*F61*)	10	ml.
Sodium bicarbonate, $NaHCO_3$	0.1	gram
Water to make	1000	ml.

Dissolve with stirring. Dispense as required. Autoclave and store.

F63.3: A, B. Phosphate-buffered dilution water (65c) A. (for utensil swabbing in restaurant inspection); B. With sodium thiosulfate (for utensil rinsing in dairy inspection)

A. Phosphate buffer, stock solution	1.25 ml.
Water, freshly boiled and cooled, to make	1000 ml.

Mix thoroughly. Dispense precisely 10.2 ml. in 15-ml. screw-capped vials. Cap loosely and tie a brown paper cover over each cap. Autoclave twenty minutes. Let the vials cool and then, without removing paper covers, screw caps on securely.

B. Phosphate buffer, stock solution	1.25 ml.
Sodium thiosulfate solution (0.1 *N*)	5 ml.
Water, freshly boiled and cooled, to make	1000 ml.

Mix thoroughly. Dispense precisely 20.2 ml. in 30-ml. screw-capped vials, or 101 ml. in 120-ml. screw-capped bottles. Cap loosely and tie a brown paper cover over each cap. Autoclave twenty minutes. Let the containers cool and then, without removing paper covers, screw caps on securely.

Phosphate buffer stock solution

Monopotassium phosphate, KH_2PO_4 (A.C.S.)	34 grams
Water	500 ml.
Normal sodium hydroxide to bring pH to 7.2	(approximately 175 ml.)
Water to make	1000 ml.

Mix thoroughly. Add a few drops of chloroform, mix well, and store.

F63.5. Glycerol solution, citrate-phosphate buffered (102)

Citric acid, $H_3C_6H_5O_7 \cdot H_2O$	1 gram
Disodium phosphate, Na_2HPO_4	13 grams
Water to make	500 ml.
Glycerol	an equal volume

Dissolve the citric acid and phosphate in water and make up to volume. Check pH, which should be 7.4. Admix the glycerol. Dispense as ordered and autoclave thirty minutes.

F63.7. Phosphate buffer solution, pH 6.6, with indicator

Disodium phosphate, Na_2HPO_4 (A.C.S.)	3.5 grams
Monopotassium phosphate, KH_2PO_4 (A.C.S.)	5.7 grams
Bromothymol blue, 0.4-per-cent solution	2.5 ml.
Water to make	1000 ml.

Dissolve the phosphates in water, add the indicator and make up to 1000 ml. Check pH, and adjust if necessary. Filter through a filter candle. Dispense as specified and autoclave thirty minutes.

F64. Sodium chloride solution, 0.85-per-cent

Sodium chloride, A.C.S. (free Ca not more than 0.005 per cent)	8.5 grams
Water to make	1000 ml.

Add water to the salt, stir to dissolve, and make up to volume. Stir well. The NaCl content may be checked by titration. Filter through hard paper. Dispense as required. Autoclave twenty to thirty minutes.

F65. Sodium chloride solution, 8.5-per-cent

Sodium chloride, A.C.S. (free Ca not more than 0.005 per cent)	85 grams
Water to make	1000 ml.

Dissolve the salt, make up to volume, and mix thoroughly. The NaCl content may be checked by titration. Filter through hard paper, dispense as required, and autoclave twenty minutes.

F66. Sodium chloride solution, borate-buffered (103)

Borax, crystal, $Na_2B_4O_7 \cdot 10H_2O$	3.5 grams
Boric acid, H_3BO_3	5.2 grams
Sodium chloride (A.C.S)	6.2 grams
Water to make	1000 ml.

Dissolve the ingredients and make up to volume. Check pH which should be 8.4. Filter through hard paper. Dispense as ordered and autoclave thirty minutes.

F67. Sodium chloride solution, with borate (104)

Boric acid, H_3BO_3 (A.C.S.)	6.2 grams
Normal sodium hydroxide (carbonate-free)	52.5 ml.
Normal hydrochloric acid	47.5 ml.
Sodium chloride (A.C.S.)	76.5 grams
Water, freshly boiled, to make	10 liters

Wash the boric acid into a liter volumetric flask with the NaOH. Shake well and add the HCl. Shake again, add about 500 ml. of the water and shake until dissolved. Make up to 1000 ml., mix thoroughly, and determine pH, which should be 7.6–7.8. Dissolve the NaCl in part of the remaining water, stir well, and make up to 9000 ml. The NaCl content may be checked by titration. Combine the solutions, determine the pH, which should be 7.6–7.8, and filter through hard paper. Dispense as required and autoclave thirty minutes.

F68. Sodium chloride solution with 0.25-per-cent or 0.5-per-cent phenol

Sodium chloride	8.5 grams
Phenol	2.5 or 5 grams, respectively
Water	1000 ml.

Combine salt and water; stir well. A check by titration may be made. Admix the phenol, filter through hard paper, and dispense as required. Autoclave thirty minutes.

F68.5. Sodium chloride solution, phosphate buffered to a specified pH

Sodium chloride (A.C.S.)	6.8 grams
Disodium phosphate, Na_2HPO_4 (A.C.S.)	see table below
Monopotassium phosphate, KH_2PO_4 (A.C.S.)	see table below
Water to make	1000 ml.

For pH	6.0	6.4	6.8	7.2	7.6	8.0	8.4
Na_2HPO_4, grams	0.30	0.60	1.05	1.48	1.69	1.85	1.90
KH_2PO_4, grams	1.53	1.24	0.81	0.43	0.20	0.10	0.02

Dissolve the sodium chloride and the phosphates in water and make up to 1000 ml. Check pH and adjust if necessary. Filter through hard paper. Dispense as specified and autoclave thirty minutes.

F69: A, B. Sodium chloride solution, phthalate-buffered (105): A. Isotonic; B. Hypertonic

Sodium chloride (A.C.S.)	6.1 or 17.6 grams, respectively
Potassium acid phthalate buffer (LaMotte)	2 grams
Normal sodium hydroxide	9 ml.
Water, freshly boiled and cooled, to make	1000 ml.

Dissolve the salt and the phthalate in a small quantity of the water, add the NaOH, and make up to volume. The amount of NaOH used should be enough to make the final pH 6.0. Adjust if necessary. Filter through hard paper. Dispense about 10 ml. in a 50-ml. flask and the remainder as required. Autoclave thirty minutes. Check pH of solution in the 50-ml. flask.

F70. Sodium chloride glycerol solution, buffered (106, 107)

Sodium chloride	4.2 grams
Monopotassium phosphate, KH_2PO_4	1 gram
Dipotassium phosphate, K_2HPO_4	3.1 grams
Glycerol	300 ml.
Water	700 ml.

Dissolve the salts in the water and admix the glycerol. Check pH and if necessary adjust to 7.2. Filter through paper and dispense 10-ml. amounts in 65- by 35-mm. jars. Cap loosely and autoclave thirty minutes. Let the jars cool and then fasten the caps securely.

F71. Sodium citrate solution, 17-per-cent

	A	*B*
Sodium citrate		
$Na_3C_6H_5O_7$	170 grams	153 grams
or $Na_3C_6H_5O_7 \cdot 2H_2O$ (U.S.P.)	194 grams	175 grams
or $2Na_3C_6H_5O_7 \cdot 11H_2O$	235 grams	211 grams
Water to make	1000 ml.	1000 grams

Dissolve the citrate in the water, heating if necessary. Mix well and make up (*A*) to volume or (*B*) to weight; determine the specific gravity at 20° (about 1.112) and adjust concentration if necessary. Filter through paper, dispense in bottles and autoclave thirty minutes. When redispensing, put about 5 ml. of the solution in a tube and test for chlorides by adding a few drops of silver nitrate and then a few drops of concentrated nitric acid. The white precipitate formed upon addition of the silver nitrate should then dissolve. Otherwise the presence of chloride is indicated.

F72. Sodium citrate solution, 2.5-per-cent

Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$ (A.C.S.)	25 grams
Water to make	1000 grams

Dissolve the sodium citrate in water and make up to weight. Filter through hard paper, dispense as ordered, and autoclave thirty minutes.

F72.5. Sodium citrate chloride solution with glucose (115)
(Alsever's solution (116) modified)

Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$ (A.C.S.)	8 grams
Sodium chloride (A.C.S.)	4.2 grams
Glucose (C.P.)	20.5 grams
Citric acid, $H_3C_6H_5O_7 \cdot H_2O$ (A.C.S.), 10-per-cent solution about	8 ml.
Water to make	1000 ml.

Dissolve the ingredients in part of the water and make up to volume. The amount of citric acid used should be enough to make the pH 6.1. Filter through hard paper. Dispense as ordered. Autoclave at 118°–120° for fifteen minutes.

F73. Sodium desoxycholate solution (108), 1-per-cent (for pneumococcus solubility tests)

Sodium desoxycholate (Riedel-deHaen)	1 gram
Water, sterile	100 ml.

Dissolve in a sterile flask.

F73.5. Sodium lauryl sulfate solution (109), 0.2-per-cent
(for pneumococcus solubility tests)

Sodium lauryl sulfate (Duponol WA, Dupont)	0.2 gram
Distilled water	100 ml.

Dissolve the lauryl sulfate in water. The solution is opalescent but this does not interfere with its use.

F74. Phenolphthalein solution, alcoholic

Phenolphthalein	1 gram
Alcohol, 95-per-cent	105.3 ml.
Water	94.7 ml.

Dissolve the phenolphthalein in the alcohol, add the water, and dispense as required.

F74.5. Cobalt chloride solution (sympathetic ink)

Cobalt chloride, $CoCl_2 \cdot 6H_2O$	2 grams
Hydrochloric acid, 30-per-cent by weight	10 ml.
Water	88 ml.

Mix the ingredients thoroughly. Test the efficiency of the mixture by writing with it on a slip of paper, enclosing this in a metal container and subjecting to dry sterilization. Writing should be visible after this treatment.

F75. Cresol solution, 1-per-cent

Cresol, (U.S.P.)	80 ml.
Tap water to make	8000 ml.

Mix ingredients thoroughly and dispense as required.

F76. Cresol compound solution, 2-per-cent

Cresol compound, saponified	20 ml.
Tap water to make	1000 ml.

Mix ingredients thoroughly and dispense as required.

F77. Phenol solution, 5-per-cent

Phenol, crystalline	50 grams
Water to make	1000 grams

Warm water to about 70°, admix the phenol, and make up to 1 kg. Filter through paper, and dispense as required.

F78A. Sulfuric chromic acid cleaning mixture

Sodium dichromate, $Na_2Cr_2O_7$	120 grams
Tap water	1000 ml.
Concentrated sulfuric acid, commercial	1600 ml.

Dissolve the sodium dichromate in the water, pour into a 3-liter flask; place the flask in a cold water bath, and add the acid very slowly, stirring constantly. Let stand in cold water bath until cool and dispense in 2.5-liter glass-stoppered bottles.

NOTE: In pouring the mixture from such bottles use a rubber stopper bored with a 10-mm. hole as spout and a suitably bent glass tube as air inlet (about 5- to 6-mm. outside diameter). This should prevent dangerous splashing. Avoid contacts with flesh or clothing. Protect the eyes, preferably with a face shield (e.g., Dockson Model No. 1120 with 6-inch clear-vision window).

F78B. Sulfuric chromic acid cleaning mixture (110–112) (for special use)

Chromium trioxide, CrO_3, saturated aqueous solution	33.3 ml.
Water	129 ml.
Sulfuric acid, commercial, 93-per-cent by weight	872 ml.

Admix the ingredients in the order given in a Pyrex Erlenmeyer flask, carefully but thoroughly, with a swing-rotary motion of the flask held freely over a sink with the mouth directed away from persons nearby. Wear a face shield or goggles, and rubber gloves and beware of spattering. The formula should yield 1 liter of mixture, and double quantities may be mixed conveniently in a 3-liter flask. There should be no great difficulty, and a precipitate formed at first should readily redissolve. Note that the *general rule* of adding the acid to the water is followed. Cautions mentioned under *F78A* apply even more in the present case, as the mixture is more potent; and like cautions should be observed in handling the chromium trioxide solution or solid which should be kept from contact with organic matter to avoid the hazard of fire or explosion, as noted on most commercial containers.

Preparation of saturated chromium trioxide, CrO_3, solution

It is convenient to obtain commercial (99.5-per-cent by weight) CrO_3 in pound lots in glass-stoppered bottles packed within protective containers. This amount of CrO_3 added to 260 ml. of water should form a saturated solution with a slight excess of the solid. Occasional stirring with a glass rod speeds the saturation, after which the supernatant liquid may be decanted to brown glass-stoppered bottles and stored in a safe place; e.g., in original containers in a second vessel (for added protection) on a sink ledge. At 20° it should contain about 62.7 per cent CrO_3 by weight (about 1.07 grams of CrO_3 per ml.). Note: The composition of the final CrO_3-SO_3-H_2O mixture (*F78B*) by weight is 2.0-per-cent CrO_3, 83.3-per-cent H_2SO_4, 14.7-per-cent H_2O; or 2.0-per-cent CrO_3, 67.9-per-cent SO_3, 30.1-per-cent H_2O. The specific gravity (20°, water at 4°), δ, should be about 1.779. If water is allowed to accumulate in the mixture (from the atmosphere or contacts with moist glassware), no permanent precipitate should form until the relative H_2SO_4 content has been reduced to less than 80 per cent by weight in the solvent, and the mixture δ to about 1.73. Within this range it is most satisfactory for use, the solubility of CrO_3 being greater at points within the range and about 2 per cent by weight at its ends (110, 111). If diluted beyond this, however, precipitation occurs down to the point of minimum solubility at about 70-per-cent H_2SO_4 in the solvent (approximately the case in *F78A*), beyond which dilution promotes CrO_3 solubility. This gives an obvious advantage to the use of supernatant liquor from *F78A*, in that subsequent removal by rinsing with water is easier, but most of the chromate there added (about four-fifths) remains precipitated and the mixture is otherwise less potent (112). The choice appears to rest with *F78A* for rapid routine cleaning of glassware difficult to rinse thoroughly, but use of *F78B* appears to offer no difficulty that is not presented in the use of cleaning fluids as practiced in most laboratories (e.g., saturated potassium dichromate in concentrated sulfuric acid), and it should be more satisfactory than the weaker preparation where special care may be taken with apparatus or it is readily rinsed thoroughly.

Reclaiming

By adding per liter the necessary amount of acid (93-per-cent H_2SO_4 by weight) and 0.0382 times as much saturated CrO_3, it is easy to reclaim the diluted *F78B* as follows:

Let δ be the first number, and the amount of the acid in milliliters required be the second number in the following pairs, (1.78, 0), (1.77, 134), (1.76, 277), (1.75, 415), (1.74, 546), (1.73, 672), (1.72, 791), (1.71, 910), (1.70, 1025), (1.69, 1136), (1.68, 1245), (1.67, 1354), (1.66, 1462), (1.65, 1569), (1.64, 1674), (1.63, 1776), (1.62, 1879), (1.61, 1980). Adjustment may be made, roughly, after a little experience by judgment of the appearance of the mixture, always adding the sulfuric acid and saturated CrO_3 in the same relative proportions (1:0.0382 by volume).

F79. Paraffin-mineral oil mixture

Paraffin, (U.S.P.)	100 grams
Mineral oil (albolene or comptol)	200 grams

Place ingredients in flask. Heat in water bath to melt paraffin and mix thoroughly. Filter through cotton. Dispense 1.5-ml. amounts in 22- by 165-mm. tubes. Warm in an oven and allow to cool after the mixture has settled. Plug and dry-sterilize.

F80. Beeswax-vaseline mixture

Beeswax	600 grams
Vaseline	400 grams

Combine, melt, and mix well. Filter through cotton and dispense as required. Dry-sterilize.

Note: This mixture is used for sealing capillary tubes in diagnostic outfits. As a grease for stopcocks, desiccators, etc., the proportions may be varied to suit conditions.

F80.2. Paraffin-vaseline mixture (113)

Paraffin, (U.S.P.)	100 grams
Vaseline	600 grams

Combine, melt, and mix well. Dispense as required.

Note: This mixture is used for sealing desiccators for use at low temperature.

F81. Dressing wax (for Alberene equipment)

Paraffin, (U.S.P.)	450 grams
Gasoline, unmodified	340 ml.
Kerosene	340 ml.

Melt the paraffin in a water bath, cool to about 60°, and add the kerosene slowly, stirring constantly. Admix the gasoline and dispense as required. Keep tightly corked and away from flames.

F82. Paper pulp

Scrap filter paper, soft	180 grams
Water to make	6000 ml.

Wash paper scraps in about 2 liters of water. Pour off water and repeat washings until clean. Pour off last wash water, add about 2 liters of clean water, and break paper into fine particles by hand. Make up to 6 liters and aerate vigorously overnight in an 8-liter bottle. Dispense in 2-liter flasks about two-thirds full, and autoclave thirty minutes.

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CHAPTER 14

STERILIZATION

In a bacteriologic laboratory the object of sterilization is either to make material safe for disposal or to provide an uncontaminated environment for transfer, study, or storage of bacterial cultures, specimens, or other materials under investigation. In the first case, advantage may be taken of knowledge of the kinds of microorganisms to be removed or destroyed, and choice of method to be used depends chiefly upon facility, provided the material is not rendered unduly difficult to handle, as by incrustation or gumming. In the second case, it is of paramount importance to select an effective sterilization technic that will be as free as practicable from detrimental effects upon the material.

Routine sterilization is effected by maintaining the material for a given period at prescribed temperatures in either a dry or moist atmosphere, by contact with chemicals, or by passage through sterile filters.

Steam sterilizers are most generally useful, particularly the rectangular double-walled (jacketed) type of the following dimensions in feet: 4 by 5 by 6, 2 by 3 by 5, and 2 by 2 by 3. They should be equipped to permit evacuation of the sterilizing chamber before admission of steam, and replacement of the steam by air under the same pressure after sterilization. The arrangement should allow independent or simultaneous control of the steam in the jacket and chamber. Other accessories should include suitable reducing valves if needed in the supply lines, thermal expansion-type traps in the exhaust lines, safety valves, and pressure and temperature indicators—the latter installed so as to show minimum temperatures in the chamber. A valve-controlled by-pass exhaust from the chamber to the atmosphere should be provided, also, to facilitate maintenance of steam at atmospheric pressure in the chamber when required, as in fractional sterilizations. If autoclaves, with doors designed to lock only with internal pressure, are to be used with initial evacuation, then additional attachments must be provided to hold the door shut. It is sometimes convenient to have steam sterilizers designed to withstand high jacket pressures to permit their use for dry sterilization. However, thermoregulated electric ovens with mechanically induced horizontal convection are usually preferable for the purpose.

An inspissator should be available for germicidal treatments at temperatures below 100°C., although other methods may be used.

Where it is necessary to avoid injury to fluid materials by heat, sterilization may be effected by filtration through sterilized candles of diatomaceous earth or unglazed porcelain, or pads of paper pulp and asbestos fiber.

Chemicals commonly used for sterilization include 1-per-cent cresol (*F75*), 2-per-cent cresol compound (*F76*), and phenol in dilutions from 0.5 to 5 per cent (*F77*). For special purposes, chloroform, which is volatile, or chlorine, which is readily removed or neutralized, may be used.

Maintaining the efficiency of the sterilizing equipment requires constant care. Exhaust lines, particularly traps and screens, should not be allowed to become congested with accumulated material such as broken glass, cotton, and dirt. They should be cleaned at least every two weeks, otherwise evacuation and sterilization may be unsatisfactory. Ground joint surfaces on the doors of autoclaves should be kept clean and undented and should never be polished with coarse abrasives. Doors with gasket seals should not be tightened unduly because the gaskets may thus be damaged.

In loading sterilizers the utmost care should be exercised to avoid undue obstruction of the circulation of the sterilizing medium by too close packing of containers or their contents. The heavier articles should be placed toward the bottom. The time interval is usually measured from the instant the prescribed conditions of temperature and pressure are attained. In the use of steam for sterilization above 100°C., enough pressure should be maintained in the chamber to prevent boiling of culture media, especially in cooling. For dry sterilization it is convenient to place certain types of apparatus such as Petri plates and pipettes in closed containers, and, as an indicator that material has been subjected to the treatment, a slip of paper is enclosed in each with the letter "S" written with cobalt chloride solution (*F74.5*). The writing should be visible after sterilization.

CONVENTIONAL STERILIZATION PROCEDURES

Dry Sterilization.—Usually one hour between 160° and 175°C. at atmospheric pressure. This is the usual sterilization procedure for prepared glassware, particularly when in tightly closed metal containers, e.g., pipettes and Petri plates. An electric oven with a fan to expedite convection is preferable; but, as noted above, a steam sterilizer may be used by admitting steam to the jacket only, provided the instrument is of sufficiently sturdy construction to withstand the relatively high pressures needed to develop the required temperature in the dry inner chamber, as indicated by table 9.

Sterilization by Steam under Pressure.—Usually at 121°C., at 2 atmospheres pressure (gauge reading, 15 pounds). This is the most common means of sterilizing culture media. It is used also for prepared glassware that might not survive the routine dry sterilization. After the chamber is loaded, all valves are closed and the door locked. The routine procedure is to evacuate the chamber as much as practicable, the degree of evacuation depending upon the temperature of the media. Table 9 furnishes a rough guide to the amount of pressure required to prevent boiling of water at the indicated temperature. After this operation is completed the vacuum line

TABLE 9

Approximate water vapor tensions

P	p	t	P	p	t
		°C.			°C.
1.5	−13.5	46	26	11	116.5
2	−13	52	27	12	117.6
3	−12	61	28	13	118.8
4	−11	67	29	14	119.9
5	−10	72	30	15	121.0
7	−8	80	31	16	122.0
9	−6	86	32	17	123.1
11	−4	92	33	18	124.1
13	−2	96	34	19	125.1
15	0	100	35	20	126.0
16	1	101.9	36	21	127.0
17	2	103.6	37	22	127.9
18	3	105.3	38	23	128.8
19	4	106.9	39	24	129.7
20	5	108.4	40	25	130.5
21	6	109.9	45	30	133.8
22	7	111.3	55	40	141.5
23	8	112.7	65	50	147.6
24	9	114.0	75	60	153.0
25	10	115.2	85	70	157.8
			95	80	162.2
			105	90	166.2
			115	100	169.9
			125	110	173.4
			140	125	178.3

P = the pressure within the system

t = the corresponding boiling point of water

p = the difference between the pressure within the system and atmospheric pressure

NOTE: The pressures are expressed in pounds per square inch. The pressure in the chamber should always be as great as the vapor pressure of water at the greatest temperature of media in the system at the time in question. Care should be exercised to note such temperatures roughly before the evacuation process and to avoid too rapid pressure reduction at the end of the prescribed sterilization period. In use of a steam sterilizer for dry sterilization, the jacket should be built to stand the pressure difference, p, indicated for the required temperatures.

is closed, and steam admitted to the chamber until the pressure equals or slightly exceeds that of the atmosphere. Then the chamber exhaust line is opened and the steam inlet opened slightly to both chamber and jacket until the atmospheric pressure is attained in each (gauge reading, zero). Then, with both exhaust lines (equipped with thermal expansion-type traps) open, both steam-inlet valves are opened wide until the prescribed pressure is reached. When the required chamber temperature is also reached, the timing of the sterilization period is begun, during which the given temperature and pressure should be maintained. At the end of the given period, the steam-inlet valves are closed, and pressure and temperature are allowed to drop slowly. However, the cooling may be expedited at this point by cautious replacement of the steam in the chamber by air under sufficient pressure to prevent boiling. When the chamber pressure again equals that of the atmosphere and the temperature is less than 98°C., the door may be opened and the treated material carefully removed. *Special caution should be observed in removing containers of liquid from the sterilizer; the liquids may still be at temperatures above the boiling point at atmospheric pressure and explosive boiling may occur. The containers should not be handled until at least ten minutes have elapsed after the opening of the sterilizer door and then with special care against agitation of the contents.*

Fractional Sterilization, Intermittent Sterilization by Steam at Atmospheric Pressure.—On three successive days the material to be sterilized is kept for a prescribed period in streaming steam at 1 atmosphere pressure (gauge reading, zero), about 100°C., usually in a steam sterilizer of the same type employed for higher pressures. During a preliminary evacuation of the chamber as above, steam is admitted (1–2 pounds) to the jacket only. Then the vacuum line is closed and steam admitted to the chamber as well as the jacket. When the chamber pressure gauge reads zero, the chamber by-pass exhaust valve is opened. When the chamber temperature reaches 98°–100°C., the jacket inlet valve is closed. The periods are timed in each instance from the moment the temperature reaches 98°C. The steam supply should be controlled to maintain the temperature between 98° and 100°C. At the end of the prescribed interval the chamber inlet valve is closed. The door is opened only when the temperature is below 98°C.

Sterilization at Temperatures below 100°C.—Usually an inspissator is used. The reservoir is about three-fourths filled with water. Careful control of temperature is necessary and, as insulation, the floor and sides of the chamber are lined with nonabsorbent cotton. Tubes of medium should be well distributed within the chamber so as to promote uniformity of treatment. Two small containers of water are placed near opposite corners of the chamber to retard drying of media or condensate water.

Heat is usually supplied by gas flame, manually regulated with regard to a thermometer inserted in the water jacket, as well as thermometers placed in the chamber near the media. A steam sterilizer may be used, maintaining the required temperature by heating with steam in the jacket only.

Sterilization by Filtration.—This technic is described under "Filtration of Prophylactic, Therapeutic, and Other Preparations," p. 806.

Sterilization by Chemical Agents.—Glass rods and pipettes if not contaminated with sporeforming pathogenic microorganisms may be made safe for further handling by soaking for several hours in a 1-per-cent cresol (*F75*), or 2-per-cent cresol compound (*F76*). Rubber stoppers may be boiled in the cresol (*F75*) solution, and dried over a flame immediately before use.

SPECIAL STERILIZATION PROCEDURES

Dry-Product Sterilization by Steam under Pressure.—Where material must be recovered moisture-free, but steam pressure sterilization is desired, as in the case of vials for antitoxins, sera, or vaccines, the material is first heated to about 100°C. as in dry sterilization in a steam sterilizer, with 2 atmospheres of steam in the jacket (gauge reading, 15 pounds). The chamber is then evacuated as much as practicable. Then the vacuum line is closed and steam cautiously admitted to the chamber so as to avoid condensation. When the pressure reaches 1 atmosphere (gauge reading, zero), the chamber-exhaust valve is opened, and pressure allowed to mount slowly to the required point (gauge reading, 15 pounds, temperature, 121°C.), the same as in the jacket. The timed interval is thus begun, at the end of which all the chamber valves are closed. Then the valve to the vacuum line is opened, and the chamber maintained at a gauge reading of about −13 pounds (−27 inches of mercury) for from five to ten minutes, after which the vacuum valve is closed and air, sterilized by passage through filters in the exhaust line, is allowed to enter until atmospheric pressure is reached; and this process is repeated after closing the chamber exhaust. Then the material is removed. Throughout the process the jacket is maintained at 121°C.

Treatment with Steam under Pressure with Entrapped Air. This procedure is employed principally for coagulation of serum (or, possibly, egg media). The material is placed in a steam sterilizer; the door and both steam exhaust valves are immediately closed and the temperature raised to 50°C. by steam in the jacket. This temperature is maintained for thirty minutes. Steam is then admitted to the chamber until it reaches a prescribed temperature, usually 80°C., which is maintained for thirty minutes. Then the chamber pressure is increased to 2 atmospheres (gauge reading, 15 pounds) by influx of steam, and if the temperature does not increase to 100°C., the exhaust valve is manipulated carefully to allow enough air to escape while still maintaining the pressure at 2 atmospheres by the steam inflow till the temperature attains 100°C. This is maintained for fifty minutes, after which both jacket and chamber steam valves are closed, the jacket exhaust valve opened, and the chamber allowed slowly to approximate atmospheric pressure and a temperature below 98°C. Then the doors may be opened and the material removed. By using a greater proportion of air in the chamber the procedure may be adapted for heat-treatment at temperatures below 100°C.

Tests of Sterilization.—Occasionally, as when contaminations occur in material that has been subjected to a routine treatment for sterilization by heat, two kinds of tests are made. First, the suspected instrument is checked with maximum temperature recording thermometers suitably placed to indicate whether or not prescribed temperatures are attained throughout the chamber. While this indicates only the maximum temperature attained at respective points during the run, the operations may be stopped prematurely in test runs so as to estimate the speed of attainment of required temperatures. More detailed and precise information, as well as a means of investigating cooling speeds, is afforded by use of suitably placed thermocouples. The second type of test consists of actual attempts to sterilize highly resistant spores of nonpathogenic microorganisms, as a pure culture dried on threads and placed in test vessels with or without culture media.

Preparation of Spore-Threads for Tests of Sterilizing Procedures.—Pieces of cotton thread, approximately 25 mm. in length, that have been dry-sterilized in Petri plates are immersed in heavy broth cultures of *Bacillus subtilis* or other nonpathogenic microorganisms that form spores especially resistant to heat. After the threads are thoroughly wet with the culture, they are placed on sterile paper disks in Petri plates and stored at 20°–27°C. for at least one week.

Before use a sample thread is removed from each lot and placed in a plate of glucose beef-infusion agar (*F35A*). Unless a pure culture similar in microscopic and macroscopic appearance to the original strain develops after an incubation period of seventy-two hours, the threads are discarded. Stored threads are similarly tested for the presence of viable spores immediately before use in a test.

To determine the effectiveness of sterilization, one spore-thread is placed in each of one or more articles or portions of medium for test. All openings are plugged with cotton, and the containers are placed in the sterilizer in locations considered least favorable; for example, in steam sterilizers, remote from the steam inlet to the chamber and in the center of relatively large or compact masses of material, such as might be encountered in practice. After the test-run, the culture media containing threads are incubated for two weeks unless growth appears earlier; threads, treated in apparatus without culture medium, are transferred to plates of glucose beef-infusion agar (*F35 A*) for incubation. If growth occurs, it is examined microscopically to establish relationship to the test-strain. Whenever necessary, studies of cultural and biochemical properties are also performed.

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CHAPTER 15

PREPARATION OF GLASSWARE

Certified volumetric or other apparatus used for exacting work such as quantitative chemical analyses or for special operations in the preparation and dispensing of biologic products, is usually cleaned by workers in the group concerned with its use. In general, all other glassware is cleaned and prepared by the central group. Such apparatus, after use, should be disposed in a way to prevent spread of infective material. In a large laboratory considerable economy may be effected by adoption of specific disposal systems, with the object of eliminating undue breakage, avoiding unnecessary sorting, and permitting more specific treatment in any sterilization required before further handling, and in cleaning. Responsibility for safe and otherwise proper disposal of both infective and noninfective glassware rests with the user. A routine service is provided for the collection and sterilization of most contaminated materials, whereas noninfective material is usually delivered to the central group by the groups concerned with its use. The cleaning processes consist of such operations as a preliminary manual removal of most of the contents or adhering material, rinsing with tap water, boiling and washing in soap or soapless detergent solution, thorough rinsing in tap and, if necessary, in distilled water, followed by drying in an oven at 110°–120°C. Then the apparatus is inspected, plugged, capped, and sterilized as may be required.

Disposal.—Suitable containers with close-fitting covers (capacity between 4 and 16 liters for small articles such as test tubes, sputum jars, and Petri plates, and up to 32 liters for larger items up to 1-liter capacity) should be available in all departmental laboratories for disposal of glassware. Articles of larger capacity than 1 liter are disposed of separately. Glassware should be stacked so as to avoid needless risk of breakage. Where it is believed that there may be danger of serious infection (see "General Bacteriologic Technic," p. 4), the apparatus should be placed directly in a suitable sterilizer by a worker qualified to handle the particular material. In order not to complicate the cleaning processes unduly, separate provision should be made for apparatus containing dye, blood or serum, feces, sputum or urine, and for Petri plates and pipettes. Care should be taken to avoid drying or precipitating material upon glassware in a way to increase the difficulties of cleaning. To this end, pipettes should be completely submerged in a suitable fluid immediately after use, if practicable; e.g., in 1-per-cent sodium carbonate if regarded as noninfective, or otherwise in 1-per-cent cresol (*F75*). Apparatus containing blood or serum should be treated similarly. Petri plates should be opened and disposed so that, in the sterilization treatment, melted gels may drain from them to the bottom of the container; porous earthenware covers should be disposed in a separate container. Discarded contaminated material is collected within twenty-four hours and sterilized promptly. Workers using apparatus in contact with materials that are known to resist the routine cleaning processes should be responsible for their elimination or conversion to a tractable form, or should designate a practical cleaning process before returning the apparatus to the central group.

Sterilization.—Whenever practicable, contaminated apparatus should be sterilized by steam under pressure (121°C.) for thirty minutes. However, porous earthenware covers of Petri plates are dry-sterilized, and pipettes, graduated cylinders, and other fragile glass articles are disinfected by intimate contact with 1-per-cent cresol for at least four hours. In sterilization of test tubes used in agglutination tests, about one gram of soap is added per liter of fluid (1-per-cent cresol) in the discard pail, and the whole heated in steam at 100°C. for thirty minutes.

Cleaning Agents.—Alkali-free soap flakes, soapless detergents, and fine-grained abrasive powder are the cleaning agents most commonly used. The soapless detergent should contain a dispersing agent such as a complex molecular dehydrated phosphate to prevent deposition of insoluble magnesium and calcium salts on the glassware. Detergents are used in 0.3- to 0.5-per-cent solution. Stronger solutions tend to hasten the clouding and etching of glassware, as does unnecessarily prolonged heating in the detergent solutions. Occasionally the use of sulfuric chromic acid cleaning fluid (*F78*) may be indicated. When using this material, workers should be protected from contact with it, particularly with regard to eyes and hands.

Cleaning Operations.—Preliminary operations that facilitate the cleaning processes include: pouring out gels while still fluid after sterilization, removal of solid material with brushes or scrapers, and removal of balsam, oil, resinous material, paraffin, vaseline, or other greasy material by soaking in a hot soapless detergent solution. Apparatus containing dyes should be segregated and given special treatment as indicated by the nature of the material. Usually, long soaking in water or in a hot soapless detergent solution will facilitate this.

The usual treatment of articles of not more than 1-liter capacity is to submerge them in water in a sink equipped for boiling. See figure 26. From 3 to 5 grams of detergent per liter are added and the water is then brought to boiling and allowed to simmer gently for from three to ten minutes as appears needed. The boiling is followed by thorough washing in the same solution, using a brush or cloth on all but the smallest articles, which are cleaned by shaking. Bottles and vials up to 50-ml. capacity are enclosed in cotton mesh bags and small articles are packed in perforated metal baskets previous to boiling. Pipettes are placed flat, with points all one way, in long perforated metal baskets or upright in tall metal cylindrical racks and submerged in the solution. During boiling, they are agitated frequently to facilitate cleaning of the bore. Tubes are washed by machine. Abrasive powders are used when necessary. Fragile glassware and articles of capacity exceeding 1 liter are not boiled but are usually treated similarly otherwise. Wax pencil marks are removed from porous earthenware covers of Petri plates with waste cotton while they are still hot from sterilization; when necessary the covers are cleaned with steel wool and tap water.

Rinsing.—Warm tap water for rinsing purposes is maintained at suitable temperature (30°–35°C.), by means of an automatic mixing valve. The washed glassware is rinsed six times successively in running tap water and then in distilled water once or twice if required, as for chemical and serologic use. The outsides of bottles and flasks of capacity greater than 500 ml. are rinsed in running tap water. Their inner surfaces are rinsed by means of an apparatus that consists of a rubber hose leading from a faucet to a metal tip perforated at the end and sides to disperse a stream of water. A ring is used to support the vessel, which is rotated above the nozzle. Small vials are rinsed on a motor-driven rotary bottle-rinsing machine. In a single rotation, vials are successively rinsed both inside and outside with hot tap water, cold tap water, and distilled water. After each of these operations the rinse water is automatically blown out with air. Tubes are rinsed before removal from the washing machines in two changes of tap water and, after removal, in distilled water if required.

Pipettes are thoroughly rinsed in running tap water. A useful technic is the following:

> The pipettes are rinsed well under the tap and then placed with tips up in a tall glass cylinder provided with a Monel metal screen near the bottom. Into the cylinder is run a strong stream of tap water sufficient to force air bubbles from the pipette bores. Rinsing is then continued by means of a simple device consisting of a glass siphon hooked over the lip of the cylinder and a slow supply of running water. The current of water through the siphon is automatically made and broken, thus alternately emptying and filling the cylinder. For proper functioning of the system, the arch of the siphon should nestle snugly into the lip, which should be deep enough so that the siphon scarcely rises above the cylinder top; and the rate of supply of water should be adjusted carefully so that the siphon flow breaks when the cylinder is emptied. (See figure 27.) All 0.2-ml. pipettes are then rinsed finally in distilled water as are other pipettes if they are to be used in chemical or serologic work.

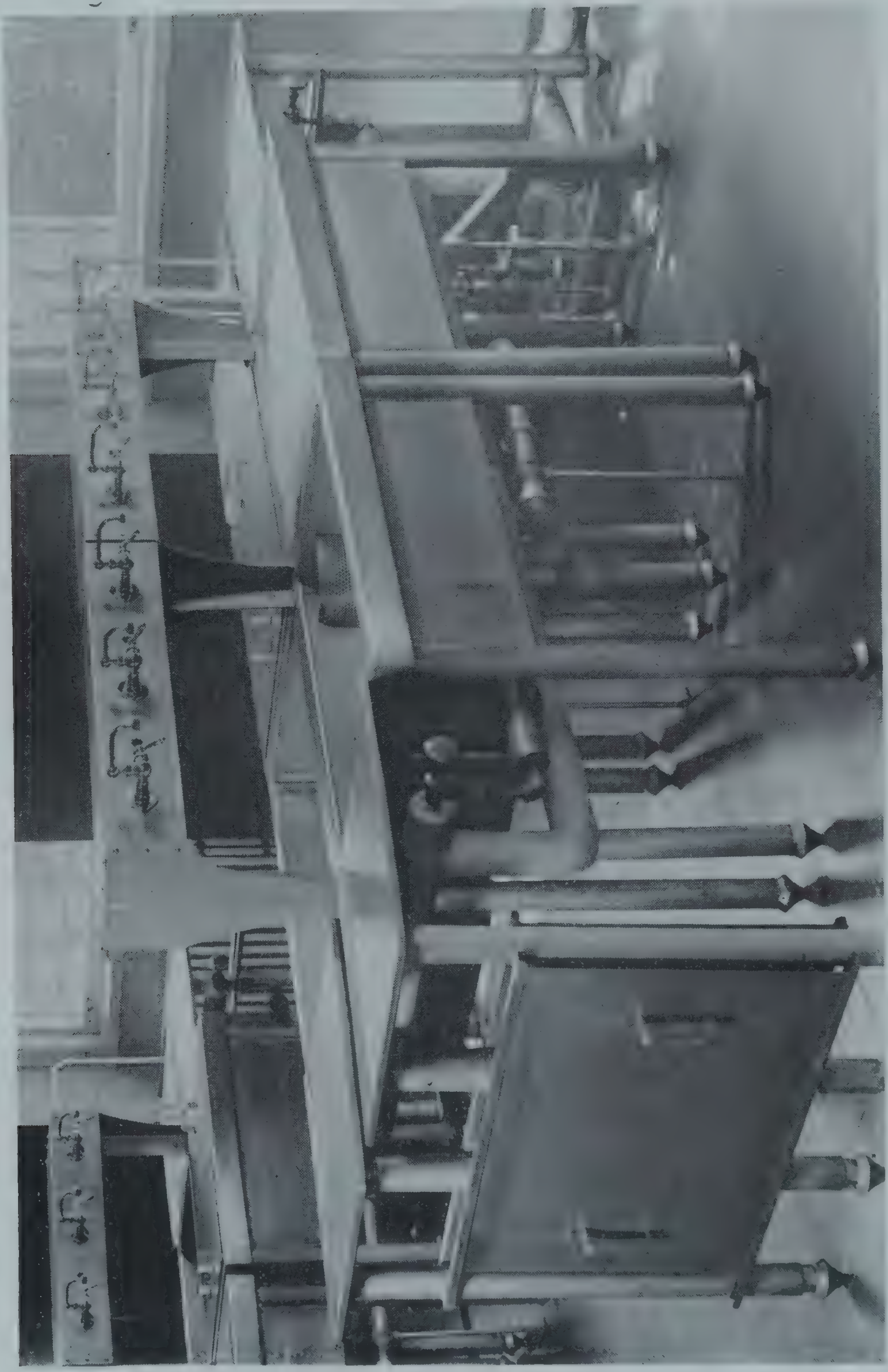


FIG. 26. STAINLESS STEEL SINKS IN SETS OF FOUR FOR BOILING AND WASHING GLASSWARE

A valve controlled steam injector is at the end of each sink under the drain tray. Visible in the nearest sink is cylinder waste strainer around the drain hole in which an overflow standpipe may be fitted.

Drying.—Usually glassware is dried at room temperature or higher temperatures up to 120°C. Wherever practicable and convenient the higher temperatures are used. Pipettes are dried thus in an inclined position with their blunt ends down. Microscope slides and cover glasses are dried by hand with lint-free cloths and by drainage or evaporation on racks.

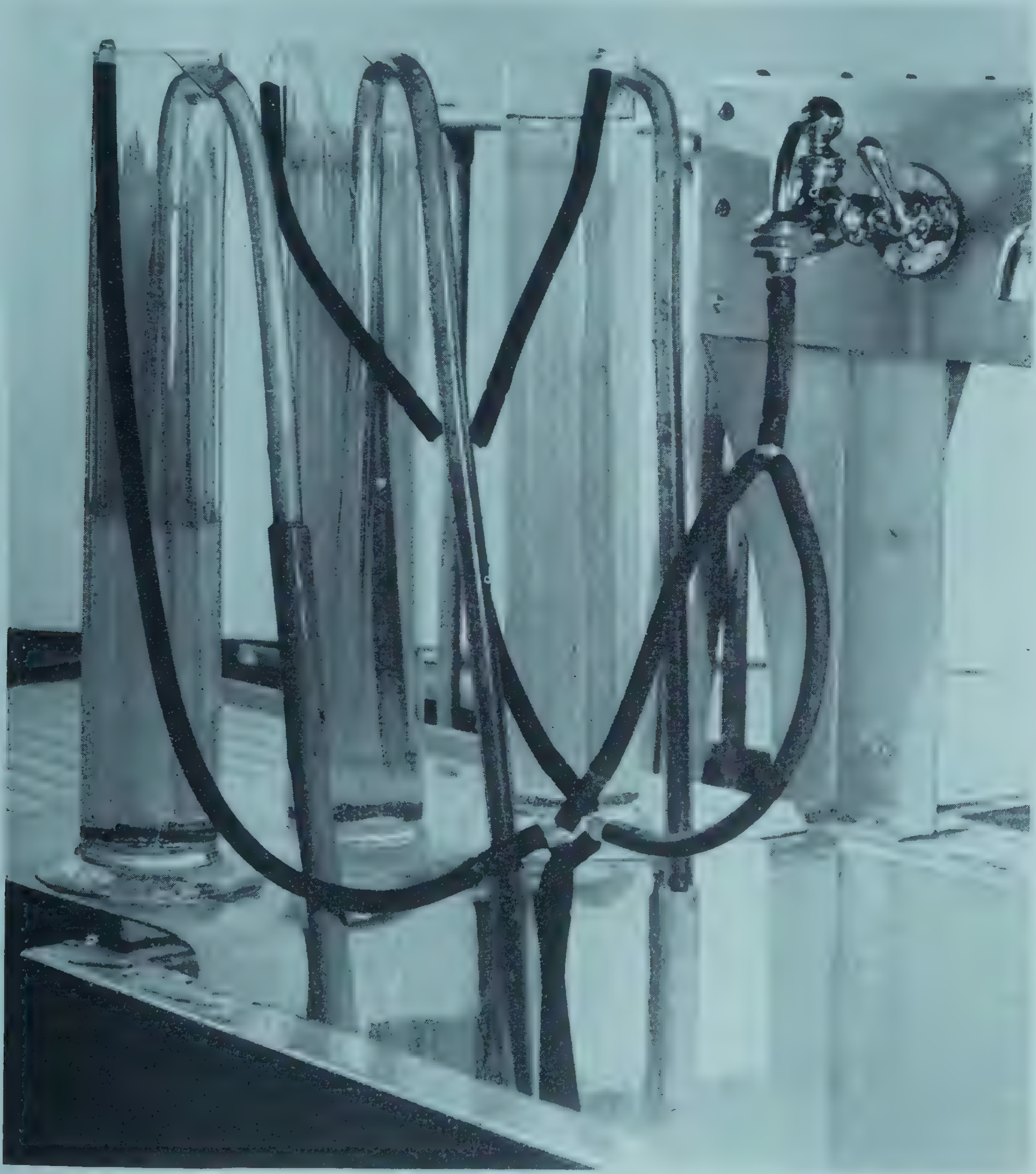


FIG. 27. PIPETTE RINSING SYSTEM

Plugging.—Care and practice are required to make satisfactory plugs. Long-fiber nonabsorbent cotton is used. A piece is separated into two or more layers, and the fibers are crossed. A reinforcement is made at the center with a small additional piece. The prepared cotton is centered over the opening to be plugged, and gently but firmly pushed into position. The plug should be evenly firm throughout, the bottom smooth and round and extending between 2.5 and 3.5 cm. into the opening, provided it does not go below the neck of the vessel. The plug should fit snugly but be readily removable with two fingers.

If films of oil form in glassware to an objectionable extent during sterilization as a result of excessive amounts of oil in the cotton, a preliminary heating of the cotton at sterilization temperatures before use in making plugs should serve as a preventive. Where special care is required to avoid scattering of cotton fibers in the glassware, the lower part of the plug is faced with or the plug completely covered with surgical gauze.

Paper or metal caps, and cork, glass, or rubber stoppers are used also when required. Special combination cotton-cork plugs for tubes are made as follows:

> The cotton is divided into thin layers (about one-quarter of the thickness used for ordinary plugs), the fibers crossed, and the center reinforced enough to make a firm butt. The cotton is pushed into the tube about half as far as for ordinary plugs, the thin upper portion flanged at the tube mouth, and the cork pushed into this so as to become surrounded by a thin layer of cotton protruding slightly from the tube.

CHAPTER 16

PREPARATION OF DIAGNOSTIC OUTFITS

Outfits assembled and distributed for the collection of specimens for microscopic, bacteriologic, serologic, or chemical examination are composed usually of sterilized apparatus sometimes containing a culture medium. They include: (1) glass slides for films of discharge or blood, (2) glass tubes (fig. 28) and jars for fluid specimens, (3) swabs and blood-letting needles encased in glass tubes for collecting and submitting specimens of blood or discharge, (4) jars containing preservative or fixative fluids, (5) tubes containing media for cultures, and (6) glass jars of different sizes for samples of water collected for bacteriologic examination of chemical analysis. They are assembled so as to facilitate return mailing in strict accordance with the Postal Laws and Regulations. Slides for films are placed in wooden cases and enclosed in heavy Manila envelopes. Single or double cylindrical mailing cases, as prescribed by the postal regulations, are used for swab, tube, and jar outfits. Directions for use and a case-history form are included.

When a specimen is packed in an outfit, the history forms should be inserted between inner and outer mailing cases whenever practicable. A code letter and color scheme for labels and history forms facilitate identification. Each specimen jar or tube bears a label for the patient's name. On tubes, this is placed as a seal with one end on the cork, to indicate to the physician about to take a specimen that the asepsis is unbroken. For detailed description, see "Outfits for the Submission of Specimens: Appendix C," p. 881.

Material of this character returned to the glassware department after use by the diagnostic laboratories is first made safe for handling by sterilization if necessary; and then reclaimed and reconditioned in so far as practicable. Mailing cases are relabeled, wooden slide cases reconditioned by sandpapering on a revolving drum, glassware and swab wires cleaned, and needles cleaned and sharpened.

Preparation of Blood-Letting Needles.—The type of needle regularly used is 19-gauge, rustless steel, fitted with a metal clip for attachment to a tube (Petroff). Needles that may be contaminated with pathogenic microorganisms are carefully removed from their mailing cases with forceps, submerged in a 2-per-cent solution of saponified cresol (*F76*) in an enamel pail, covered and sterilized at 121°C. for fifteen minutes. Noninfective needles are submerged in the cresol solution for overnight or longer but not heat-sterilized. All needles are then treated according to the following procedure.

The needles with stylets still in are rinsed and then boiled in a 0.5- to 1.0-per-cent solution of mild soap flakes for fifteen minutes after which the stylets are removed with forceps. Needles and stylets are boiled again separately in fresh soap solution. The stylets are rinsed in six changes of hot water, dried, and reused as far as practicable. The needles are placed in a shallow pan and rinsed thoroughly in six changes of hot water. Then by means of a vacuum line double-trapped with a collecting

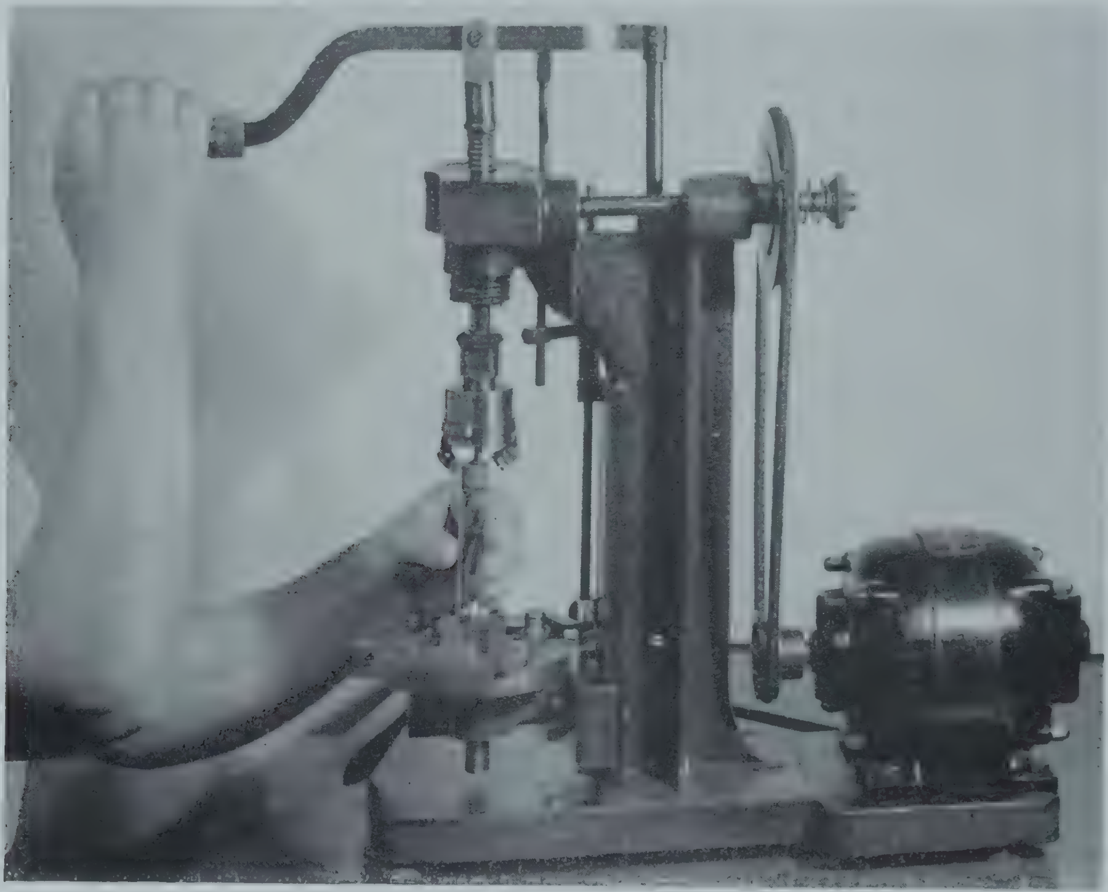
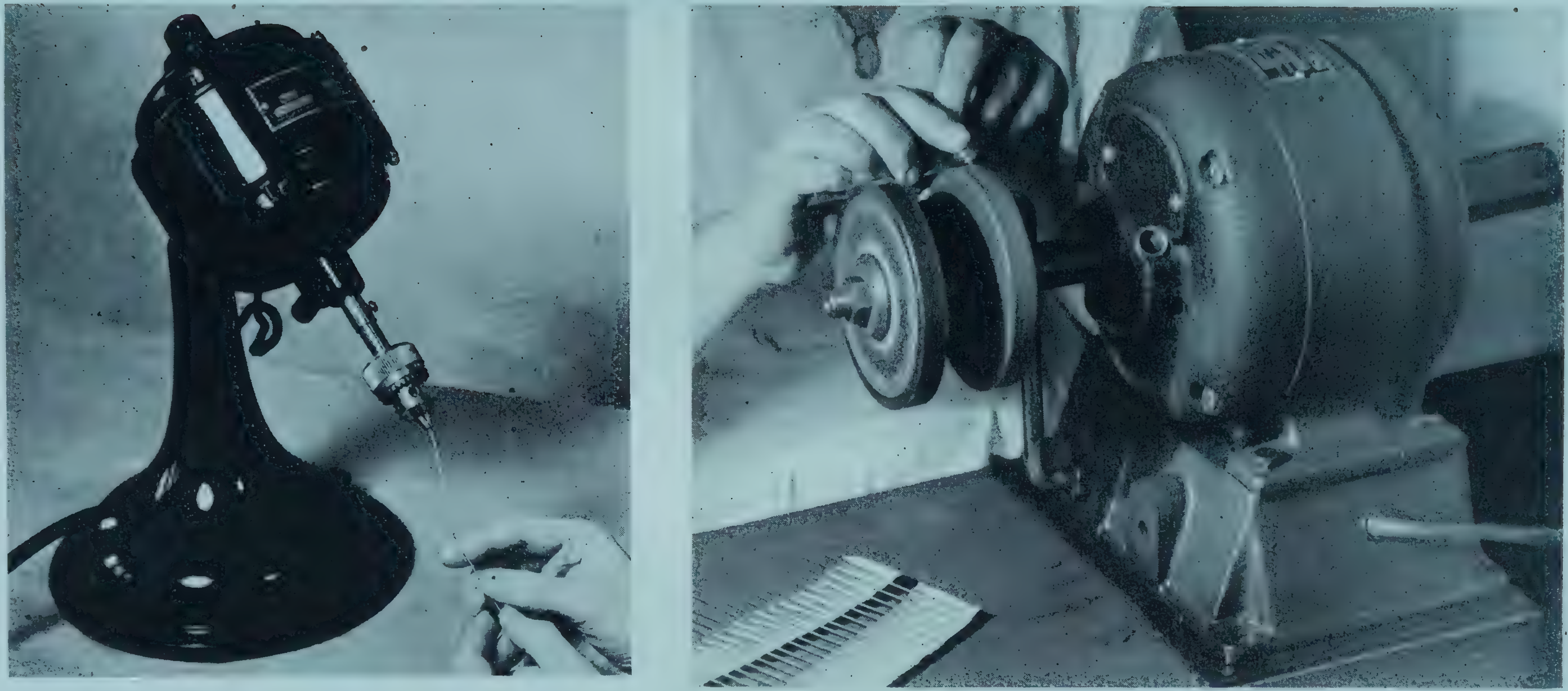


Fig. 28. Adjustable Corking Machine

This machine was designed in the laboratory machine shop primarily to provide a safety device for the corking of tubes used in diagnostic outfits. It consists of a belt-driven, automatic chuck, which when pressed down upon the cork in a test tube, grips it and twists it into place. This chuck is raised and lowered vertically by means of a lever. An automatic adjustable spring-operated chuck, fastened to the base plate of the machine, clamps the base of the tube and centers it beneath the belt-driven chuck. The depth and tightness of the cork in the tube are controlled by an adjustable spring, which presses together the friction surfaces of a compensating pulley and permits them to slip when just enough pressure has been applied. The mechanism is operated by a 1/10 h.p. motor. (Specifications and drawings, 1931. Unpublished.)



Piano-wire Drill for Plugged Needles. A Convenient Adaptation of the Stand and Motor of An Electric Fan

Needle-Sharpening Machine

Fig. 29.

flask and a glass adaptor fitted with a metal tip which has an opening just large enough to admit the pointed end of a needle, hot water is drawn through the lumen of each needle until it runs clear. Air is then allowed to flush out the water.

Clean, unplugged needles are dried quickly and thoroughly at from 110°–120°C. in an air oven, then sharpened by successive grindings on motor-driven wheels of alundum and Pike Arkansas stone, respectively (figure 29). Care should be taken to leave no burr. The lumen is finally cleaned with a stylet and then a clean stylet is inserted and bent so as to protect the point. New needles are boiled in several changes of soap solution, rinsed as above, dried, cleaned, and equipped with stylet. Needles found plugged in any of the above operations are either rejected or reamed out with a piano-wire drill (see figure 29) and then recleaned.

For complete descriptions of outfits, including those for the collection of samples of water for chemical and bacteriologic examination, see "Appendix C," p. 879.

METHODS USED IN THE DIAGNOSTIC LABORATORIES

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METHODS USED IN THE DIAGNOSTIC LABORATORIES

INTRODUCTION

The activities of the diagnostic laboratories include the examination of specimens as an aid in diagnosis; research and investigation concerned with the improvement of methods and the general advancement of the work; and the approval and maintenance of standards in the local laboratories. The requirements of the Public Health Law, the regulations of the Sanitary Code, and the policies governing approval of laboratory service are given in the Appendix.

The character of the work is constantly changing as new problems arise. Programs to eradicate syphilis, gonorrhea, and tuberculosis have necessitated more intensive study of methods in the examination of specimens for evidence of these infections. Increased interest has also been shown in the prevention, diagnosis, and treatment of syphilis of the central nervous system, thus requiring the performance of all the essential tests that show evidence of abnormalities in the cerebrospinal fluid. The study of specimens for Vi agglutination and the typing of *Bacterium typhosum* with bacteriophage have proved of marked epidemiologic value in determining the source of infection in typhoid fever. The importance of the various species of *Salmonella* as incitants of enteric disease has been particularly emphasized within recent years. At least 150 species belonging to this genus have been recognized. The identification of these microorganisms is complicated but the data obtained are of special value to the epidemiologist.

As a result of the war, the facilities of the laboratories must now be augmented to provide aids in diagnosis in a much wider field than heretofore. Diseases that formerly occurred only rarely in this country will probably be introduced and those already existent may present new problems in public health. Bacillary and protozoan dysentery, malaria, and diseases incited by helminths, fungi, and viruses may be expected to assume new relationships and significance.

With large increase in the average life expectancy, a greater proportion of the population has reached that period of life when diseases other than those infectious in origin attain a prominent position as causes of death. It is in the neoplastic diseases that pathologic diagnosis is most important as a measure of control. Moreover, they constitute one of the most difficult problems for the laboratory, since not only diagnosis but often prognosis and therapy depend on pathologic examination. There are two methods of ensuring accurate pathologic diagnosis of cancer, one by centralization of material and the other by utilizing numbers of institutions staffed by individuals competent in pathologic diagnosis. The second is preferred because it enables the clinician to establish personal contacts with the pathologist and permits him to discuss his problems at length; hence both are benefited to a degree not possible otherwise. It was with these latter ideas that the system of voluntary approval was extended to surgical pathology in 1933.

Another problem is to ensure to pathologists access to material that will maintain interest and experience at a high level. An effort is therefore being made to establish a large teaching and museum collection for instruction and review. To develop this collection, recourse has been had to the material of some of the larger hospitals, particularly the Memorial Hospital for Cancer and Allied Diseases in New York City. It is highly desirable that this educational program be amplified along certain other lines, especially forensic and industrial medicine. Interest in surgical pathology has been stimulated by the institution of an annual conference under the auspices of the New York State Association of Public Health Laboratories. These conferences have been held since 1940. They serve as a source of rare and unusual material which, it is believed, will stimulate and increase diagnostic skill.

The facilities of the central laboratory are available for consultation service in diagnostic problems. Directors in local laboratories are urged to employ these facilities and by the submission of unusual material to add to the study collection at the central laboratory.

CHAPTER 17

GENERAL INSTRUCTIONS

Records and Record Forms

Specimens for laboratory examination are accompanied by history forms supplied in mailing outfits (see "Appendix C," p. 879). These forms are maintained as the original record of the handling of the specimen. All steps in the examination and the final results are entered on them and signed with the last name of the examiner. When insufficient space is available, special forms prepared for records of additional examinations are used and stapled to the original history. Accession books are kept in which a record is made of every specimen received. The data that are recorded vary depending upon the type of examination.

Records of special studies that may be undertaken are made on 4- by 6-inch cards, which, when the work is completed, are transferred to the Scientific File, in the library and publications department. The history forms bearing records of the routine examinations are filed in the office.

Always record observations of tests and results of examinations directly on permanent forms; do not copy from notes. Never obliterate or erase records. If an error is made, indicate the necessary correction and add an explanatory statement.

General Procedures

Under the chapter "General Bacteriologic Technic," are outlined directions for handling infective material, the general bacteriologic procedures used throughout the laboratory, the technic of isolation and study of pure cultures, and conventions in general use. Procedures that are only occasionally performed in this laboratory are outlined briefly and references are given to publications that should be consulted before such examinations are undertaken.

DISPOSITION OF SPECIMENS

Keep a record of the date and the hour of receipt of all specimens. Distribute them promptly to the groups performing the examinations indicated by the mailing cases. Colors and symbols have been used to aid physicians in selecting the appropriate outfit and to facilitate the handling of specimens upon arrival at the laboratory. A complete description of each outfit and a copy of the history form accompanying it is given in "Appendix C." Medico-legal work is, in general, not undertaken since no provision is made for members of the staff to act as expert witnesses and because the schedule under which specimens are handled by a number of different workers is incompatible with the procedure prescribed for obtaining data that can be used in court.

Open and number the specimens one at a time, in order to avoid the possibility of interchanging them. (When large numbers of specimens of one type are received, the groups of persons opening them should work independently.) The first of each year, begin the laboratory number for each type of examination with "1" and number the specimens in serial order as they are opened. To avoid duplication or omission of numbers, always enter the serial number in a notebook or an accession book under the date of receipt before stamping it on the history form, on the label of the specimen tube or jar, and on the mailing case. Stamp the date on the history form.

As promptly as possible after the specimens are numbered, record in the accession book whatever data are required, such as the patient's name and the doctor's name and address. When the study is completed, record the results and the number of examinations performed. (See "Methods Used in the Administrative Offices," p. 840.)

Evaluate the data accompanying each specimen and make all examinations that may prove helpful in diagnosis.

Record in ink on the history form or on a record slip to be attached to it, the character and amount of each specimen, the procedures used, and the findings obtained on examination. The history slip and attached forms are maintained as the original records of the physician and laboratory for the number of years prescribed for each. Do not make the first record elsewhere and later transfer it to the record slip. The only exception to this procedure is in the serologic tests for syphilis and tuberculosis where the original records of the tests are recorded directly in the accession book by the examiner.

On the etched ends of glass slides, write with pencil the serial number, preceded by a letter when necessary to designate the type of examination that is being made, the year, and any other information necessary to identify the preparation. For example, if a specimen to be examined for *Neisseria gonorrhoeae* is received in 1945 and is given the serial number 1001, the slide would be labeled as in fig. 30.

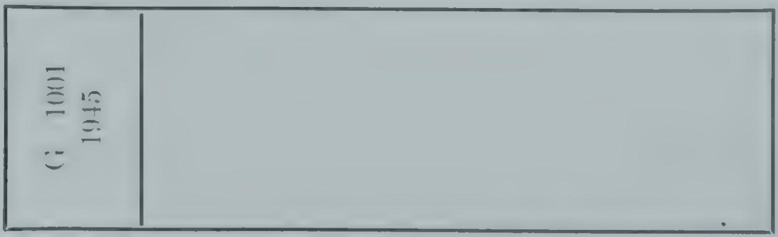

FIG. 30. SLIDE LABELED FOR STAINED PREPARATIONS

Never discard a specimen until the examination has been completed and the results reported.

When unsatisfactory material is received and the accompanying history slip indicates that the examination is urgent, advise the physician by telephone or telegram.

Keep on permanent file slides prepared from specimens for histologic examination and those of interest for the museum. File all others for nine months.

BACTERIOLOGIC EXAMINATION

Microscopic

Make a microscopic examination to provide information essential to the choice of cultural methods and for reference, that is, for comparison with the results of cultural examination or animal inoculation. Unless otherwise indicated, stain a slide preparation by Gram's method (p. 35) or with aqueous alcoholic methylene blue (p. 38), or one by each procedure. If an examination for tubercle bacilli is to be made, stain a film by the Ziehl-Neelsen method or a satisfactory modification (p. 43).

The study of unstained moist preparations may also be helpful. The use of the dark field in some instances may be particularly desirable.

Cultural

When a general bacteriologic examination is to be performed, inoculate a blood-agar plate (*F43A*) and incubate it in the presence of 5–10 per cent carbon dioxide. This method of incubation is satisfactory for the cultivation of aerobic pathogenic bacteria and especially favorable for some types of microorganisms, such as *Brucella abortus*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae*. The inoculation of semisolid agar (*F32C* or *F32D*) often facilitates the isolation of microorganisms that develop slowly on the surface of solid medium. Special media that may be required for individual species are designated in the respective chapters.

When microorganisms produce spreading growth, streak plates of medium containing potassium tellurite (*F40B*). This medium inhibits the growth of many species of bacilli that spread or "swarm," but affects very little the growth of molds or Gram-positive cocci. Poured plates made from a series of dilutions of the material may also be helpful.

Employ anaerobic methods when the clinical data or microscopic examination suggests the presence of bacteria that develop only, or more rapidly, under anaerobic conditions. Anaerobiosis is usually obtained by the incubation of cultures in a hydrogen jar. Chopped-meat medium (*F6*) and deep glucose agar (*F35A*) may also be inoculated. Incubate these media for at least two weeks unless significant findings are obtained sooner or the presence of contaminating microorganisms renders further examination useless.

After incubation, fish colonies to suitable media, as indicated by the colonial and morphologic characters. Also, make Gram-stained preparations of all the cultures isolated. Perform further tests, if necessary for identification, as outlined in the procedures for the systematic study of microorganisms in "General Bacteriologic Technic" and in the chapters dealing with the particular species.

Serologic

Whenever possible, confirm the results of microscopic and cultural study by agglutination or other serologic tests in appropriate serum. Make serologic tests with patient's serum, if available, especially when the microorganisms are of questionable diagnostic significance. Perform agglutination and precipitation tests and record the reactions as described in "General Bacteriologic Technic," p. 19.

Perform a macroscopic tube-agglutination test, employing two or three dilutions of serum in which agglutination is obtained only with the homologous species. The specific dilutions are determined by testing each new lot of serum with the homologous and all antigenically related species. Unless otherwise specified, test with broth cultures or suspensions in salt solution of growth from solid medium and adjust the turbidity to that of glass standard No. 2.5 (p. 26). If possible, avoid the use of media containing carbohydrates or other fermentable substances. Highly pathogenic microorganisms should be killed by heat or by the addition of formalin.

Include the usual controls: (1) the culture combined with salt solution and with appropriate dilutions of serum from a normal animal of the species in which the agglutinating serum was produced and (2) the highest serum dilution combined with a representative strain of the homologous species.

When living microorganisms are present, flame the tops of the tubes and plug them with nonabsorbent cotton. Shake to ensure thorough mixing, and, unless otherwise specified, incubate the tests for fifteen to twenty hours at 48°–52°C. and record the reactions.

Precipitation tests, macroscopic-slide agglutination tests, and tests for absorption of agglutinative properties may sometimes be of value in the identification of the culture.

Animal Tests

Animal tests are made when proof of toxigenicity or virulence is necessary for the identification of the microorganism, or when the inoculation of susceptible animals is desirable to establish the presence of a virus or a microorganism that cannot otherwise be demonstrated. In general, studies requiring animal inoculation can be undertaken most satisfactorily when there is opportunity for direct supervision of the collection of the specimens. (See "The Use of Experimental and Test Animals, p. 73.")

BLOOD

See also "Examination of Blood," p. 502

Cultural Examination

If the blood was collected following the administration of a sulfonamide or penicillin, use a semisolid medium (*F32C* or *F32D*) to which has been added *p*-aminobenzoic acid or its sodium salt, or a penicillin inactivator, respectively.

When clotted blood is to be examined, remove the serum for serologic tests using a capillary pipette fitted with a small rubber bulb. Follow the technic recommended by Sellers (1) for comminuting the clot: Place the barrel of a 10- or 20-ml. Luer syringe in an upright position in a jar. Pour into it the clot from the specimen tube. With the plunger, force the clot through the nozzle of the syringe into a bottle containing 50 ml. of semisolid agar (*F32C* or *F32D*). Inoculate two blood-agar plates (*F43A*) and make a film preparation from the drop of blood remaining in the tube. Incubate one plate in the presence of 5–10 per cent carbon dioxide and the other anaerobically. Stain the film with aqueous alcoholic methylene blue (p. 38).

When blood containing an anticoagulant is examined, distribute 5–10 ml. of the blood in each of two bottles containing 50 ml. of semisolid agar (*F32C* or *F32D*), 1–2 ml. in a tube of glucose beef-infusion agar (*F35A*) for a poured plate, and a few drops to the surface of each of two blood-agar plates. Unless the examination is undertaken immediately after collection of the specimen, stain slide preparations with methylene blue. If large numbers of microorganisms are present, the Gram stain (p. 35) may also be helpful. Incubate one of the blood-agar plates anaerobically and the other in the presence of 5–10 per cent carbon dioxide.

To distribute a specimen received in a Keidel tube or other container with a constricted opening, draw the blood into a capillary pipette or a 20-ml. syringe fitted with a 17- or 19-gauge needle.

If arrangements can be made to have medium inoculated directly after collection of the blood with a syringe, use 100-ml. bottles fitted with Bakelite screw caps and rubber diaphragm and containing 80-ml. of semisolid agar (*F32C* or *F32D*). After removing the cellophane cap, puncture the rubber diaphragm with the needle, and force 5–10 ml. of blood into the bottle. Withdraw the needle, mix the blood and medium by rotating, and incubate as soon as possible.

Examine semisolid agar inoculated with blood after incubation for twenty-four to forty-eight hours. If no growth has been obtained on the original plates, make another set and a film from the semisolid agar. Replate at the end of the first week and at the end of the month unless growth has occurred previously. Incubate all plates for at least seven days.

Serologic Examination

Separate the serum from the clot or blood cells by centrifugation for five minutes at approximately 1000 r.p.m. This may be done in the tube in which the specimen is received or in the centrifuge tube to which the serum and cells have been transferred. Remove the clear serum with a capillary pipette. Make any tests requested and, in addition, others that might furnish significant information. Methods for complement-fixation tests are outlined in the chapter "Serologic Tests for Evidence of Syphilis, Tuberculosis, and Gonococcus Infection," p. 361.

If the blood is hemolyzed or the serum cloudy, make a stained preparation to determine the presence of contaminating microorganisms. If no bacteria are found or if those present resemble pathogenic species in morphology, culture the clot. Contaminating microorganisms or exposure to extreme heat or cold, which also produces hemolysis, may affect the agglutinative properties. Consider of questionable significance the results of the examination of specimens that are apparently chylous, those to which an anticoagulant has been added, and those received five days or more after collection. Always examine the serum, however, unless its condition is such that it would obscure the reaction.

Specimens of whole blood should be requested for serologic tests. Hemolysis may occur if specimens are contaminated, overheated, frozen, or otherwise rendered unsatisfactory for examination. A similar condition in a specimen of serum is less readily detected. Thus, in general, the results of the examination of serum should be considered of questionable significance. However, in certain examinations, for example the agglutination test, an equal volume of glycerol C.P. acts as an efficient preservative for serum. Specimens containing glycerol are not satisfactory for complement-fixation tests.

Macroscopic Tube-Agglutination Test.—Perform macroscopic tube-agglutination tests as outlined here and in "General Bacteriologic Technic," p. 19. Test a series of dilutions of serum usually beginning with 1:5 or 1:10. It is generally convenient to prepare only the required amount of each dilution in 11- by 75-mm. tubes in which the tests are incubated. Include controls consisting of each culture or suspension employed combined with (1) salt solution and (2) a specific dilution of known serum. When the specificity of the suspension has not been established, test it with sera from two or more apparently healthy persons. Further details will be found in chapters dealing with individual species.

When a survey is undertaken to determine the incidence of agglutination reactions with a given microorganism or for any other reason large numbers of specimens are examined, time and materials may be saved by performing preliminary tests with one low dilution of each serum (1:20–1:60) (2). Pipette sufficient serum into each tube to give the approximate dilution when combined with 0.6 ml. of agglutinating suspension; for example, 0.03 ml. of serum for a 1:20 dilution or 0.01 ml., for a 1:60 dilution. Use a suspension having a turbidity one-half that of glass standard No. 2.5. When reactions are obtained, test a series of dilutions to determine the titer.

Unless otherwise specified, report the dilution in which complete or nearly complete agglutination (4+ or 3+) occurs. When agglutination is obtained only in low dilutions, no greater than 1:40, report a partial reaction and add a statement that its significance may be questionable. Advise that another specimen be submitted for confirmatory examination.

Centrifuging the tests for ten minutes at 2000 r.p.m. often demonstrates agglutination where it was not apparent before. When this occurs, repeat the test and report the findings, advising that another specimen be submitted. Centrifuging after incubation for fifteen minutes may be helpful when an early report is needed and the results of the standard test cannot be reported within twenty-four hours, as on week ends or holidays (3–6).

URINE

Examinations for tubercle bacilli or other microorganisms can be undertaken most satisfactorily when the specimens are collected with aseptic precautions and examined promptly. The procedures to be followed will depend upon the clinical data and the results of microscopic examination of the sediment.

See also "Examination of Urine," p. 513.

Microscopic Examination

Centrifuge; stain films by the Ziehl-Neelsen, or a satisfactory modification (p. 43), and Gram (p. 35) methods. (See "Examination for Mycobacteria," p. 292.)

Cultural Examination

Unless aseptic precautions have been observed during collection, the specimens are generally unsatisfactory for cultural tests.

Streak a small amount of the sediment on blood agar (*F43A*). Identify any bacteria that are present under conditions which suggest that they may be of diagnostic significance. Cultural examinations for this purpose are considered in chapters dealing with the microorganisms concerned.

CEREBROSPINAL FLUID

S e "Examination of Cerebrospinal Fluid," p. 517.

PLEURAL, ASCITIC, AND OTHER FLUIDS

Microscopic Examination

If the history suggests infection with tubercle bacilli, centrifuge the specimen and make slide preparations from the sediment. Stain one by Gram's method (p. 35), one with aqueous alcoholic methylene blue (p. 38), and two by the Ziehl-Neelsen method, or a satisfactory modification (p. 43). (See "Examination for Mycobacteria," p. 292.) Animal inoculation is undertaken when the clinical history indicates that it may be an aid in diagnosis.

Cultural Examination

If the examination of stained preparations or the clinical data indicate the presence of microorganisms other than tubercle bacilli, try to isolate and identify them according to the procedures outlined for bacteriologic examination.

PURULENT DISCHARGES

Follow the procedures described for bacteriologic examination and identify the microorganisms found. Special cultural work or animal inoculation may be required, depending upon the clinical data or the results of bacteriologic tests.

STOMACH AND DUODENAL CONTENTS

Children and sometimes older patients may swallow sputum. Consequently, the examination of stomach washings for tubercle bacilli, pneumococci, or other pathogenic bacteria may be desirable.

A study of duodenal contents as an aid in determining the localization of infections of the gall bladder may be important. For examinations for typhoid bacilli and *Salmonella* see "Examination for Evidence of Infection with Bacterial Incitants of Enteric Disease," p. 307.

Other types of examinations of gastric and duodenal contents are seldom made unless the work can be undertaken soon after the material is collected. When such specimens are received in a condition satisfactory for the tests desired, follow the technic described in standard textbooks on clinical pathology.

FECES

For examination of feces for bacillary incitants of enteric disease, see "Examination for Evidence of Infection with Bacterial Incitants of Enteric

Disease," p. 297. Enteric diseases induced by animal parasites may be broadly divided into those incited by protozoa and by various intestinal worms or helminths. In examinations for evidence of these parasites, consult the specific chapters.

The microscopic appearance of the feces is often helpful in differentiating amebic from bacillary dysentery. (See "Examination for Protozoa," p. 479.) When gross evidence of the presence of blood is not found, a benzidine test may sometimes be desirable. (See "Examination of Urine," p. 513.)

REPORTING RESULTS

As soon as significant results are obtained, write a report on the history slip in such form that it may be typed and sent to the physician or, if advisable, report the results in a letter.

Report the results of preliminary examinations only when the available data will not be misleading, and indicate that further work is being done.

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CHAPTER 18

EXAMINATION FOR PNEUMOCOCCI

Although modern therapy has removed the urgency for pneumococcus-type differentiation, the information provided by this examination is still required in the event that the progress of the patient indicates the need for instituting serum treatment.

Pneumococcus-type differentiation can be undertaken with sputum from the deeper air passages, and with blood, cerebrospinal fluid, pleural and other exudates, and stomach washings.

Sputum is sometimes collected from a child by swabbing the back of the throat during the act of coughing.

SPUTUM

Examine the specimen immediately by the microserologic technic of Neufeld (1) except when it is received after laboratory hours and the accompanying data do not indicate that a prompt report is essential to the management of the case. Under such circumstances, defer the examination until the following day. Report at once characteristic reactions in preparations with any of the type-specific sera. When a pneumococcus type is not recognized by this method, prepare films, streak a plate of blood agar (*F43A*), and inject a mouse. Request another specimen, especially if for any reason the first is not entirely satisfactory; for example, if it is insufficient for examination or appears to consist of saliva.

Examine sputum submitted for pneumococcus-type differentiation for tubercle bacilli also, p. 292.

If the amount of sputum available is insufficient for the microserologic test, emulsify in beef-infusion glucose broth (*F5A*) and streak a plate of blood agar. This broth may be tested by the microserologic method after incubation for eighteen to twenty hours.

Specimens from the throat or nasopharynx of a high percentage of individuals contain pneumococci, and not infrequently more than one type is present. Consequently, when considerable numbers of cocci having the appearance of pneumococci are found in sputum from patients with pneumonia, and capsules are demonstrated on only a few of them with one of the monovalent sera, it is important to examine the specimen for other types of pneumococci. In sputum from the bronchi, the incitant of the infection will usually be present in greater number than the others while the reverse may be true of specimens consisting largely of post-nasal discharge.

Microscopic Examination

Microserologic Test (**Quellungsreaktion, Neufeld**) (**1–3**).—Select a purulent fleck of sputum, avoiding liquid portions and mucus. Mix on a slide with two or three times its volume of serum and one or two times its volume of alkaline methylene blue (p. 37). Various sized loops or small capillary pipettes are convenient for transferring the material. Apply a cover glass, and, if the preparation is to be kept for some time, seal with vaseline. If slides 5.1 by 7.6 cm. and cover glasses 1.8 cm. square are employed, six preparations can be tested on each slide, see fig. 31. Use a microscope lamp that provides an intense illumination and examine with the oil-immersion objective. Adjust the iris of the condenser to give maximum definition. The capsular swelling usually occurs promptly. However, if this reaction is not obtained immediately, re-examine the preparations after thirty minutes.

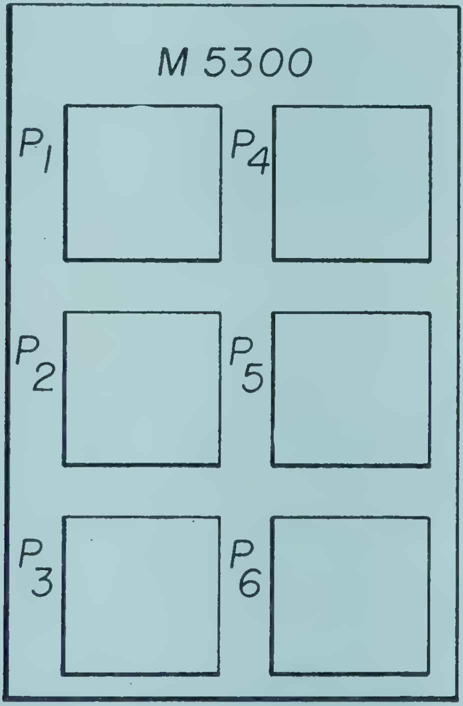

Fig. 31. Slide, 5.1 by 7.6 cm. with Six Preparations for Microserologic Examination for Pneumococci

The bacteria and nuclei of pus cells appear deep blue against a pale blue background. The capsular swelling is apparent as a gray-green refractile area with a clearly defined periphery. See fig. 32. The size of the swollen capsules varies on different types and strains of pneumococci. For example, those on types 2, 3, and 8 may be very large while those on type 5 are usually small. In sera prepared with heterologous types, the capsules are either invisible or form only a narrow poorly defined halo surrounding the microorganism. See fig. 33.

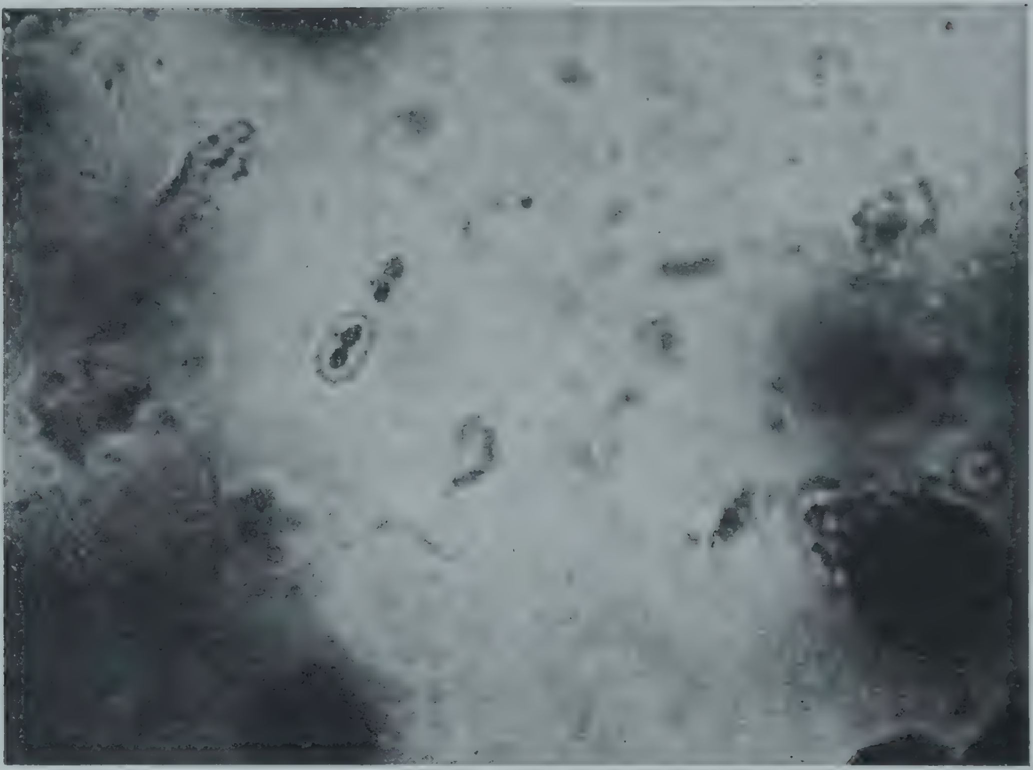

FIG. 32
PHOTOMICROGRAPH OF SPECIMEN OF SPUTUM SHOWING PNEUMOCOCCI TREATED WITH SERUM OF HOMOLOGOUS TYPE (× 1500)

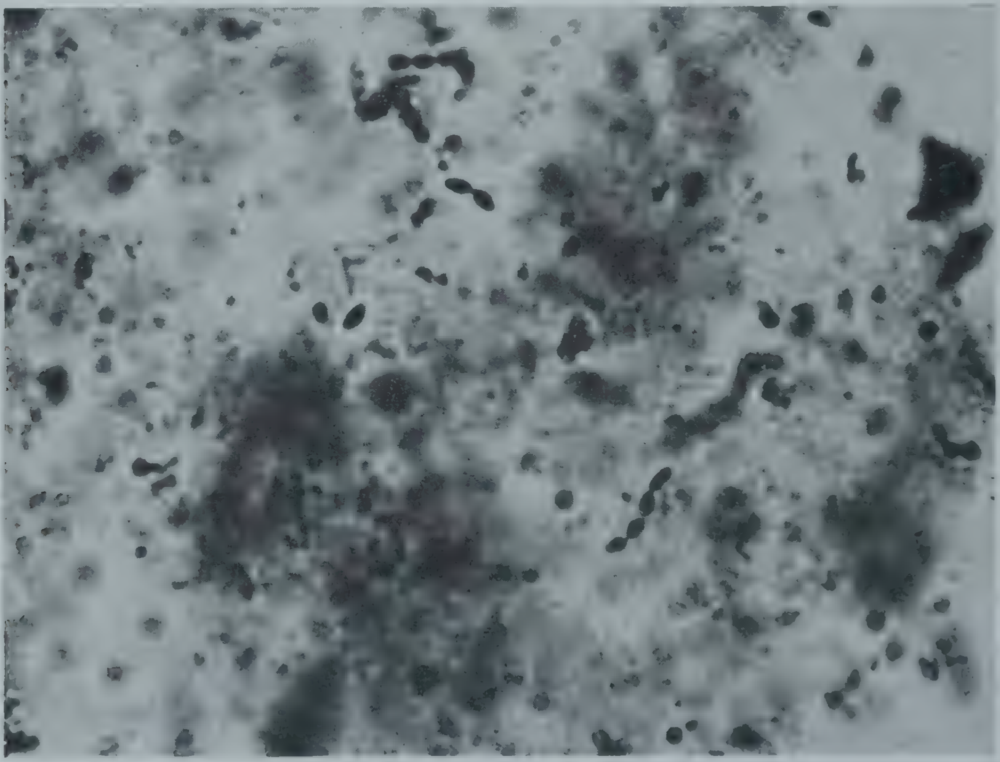

FIG. 33
PHOTOMICROGRAPH OF SPECIMEN OF SPUTUM SHOWING PNEUMOCOCCI TREATED WITH SERUM OF HETEROLOGOUS TYPE (× 1500)

First examine for capsular swelling in pools of sera prepared by combining four to seven univalent rabbit sera. (See methods for the production and standardization of antipneumococcus sera for pneumococcus-type differentiation, p. 788). When a reaction occurs, test the sputum with each serum included in the pool.

When capsular swelling is not demonstrated in any of the pooled sera, stain films by Gram's method (p. 35), since, as in other bacteriologic procedures, the results of microscopic examinations are helpful as a guide for further tests. When cocci having the morphology of pneumococci are present, test with sera for types other than 1 through 33 that are not included in the pools (4–7). A further study should be made of any strain that reacts irregularly in the available sera.

If a few pneumococci of one type and a considerable number of another type are found in sputum, their significance requires careful evaluation. The examination of blood or of a second sputum specimen may be very helpful.

When specific capsular swelling is demonstrated, confirmation by other procedures is not undertaken for diagnostic purposes.

Cultural Examination

Streak blood agar plates with sputum and incubate in the presence of 5–10 per cent carbon dioxide. Examine after eighteen to twenty hours' incubation for small, umbilicated, colorless colonies surrounded by a zone of green discoloration. With a needle or loop, dissect small blocks of the blood agar containing such colonies and transfer them to tubes of beef-infusion-glucose broth (*F5A*). Study the morphology of the microorganism in the broth after eighteen to twenty hours' incubation, test for solubility in bile, sodium desoxycholate, or sodium lauryl sulfate (p. 18), and make microserologic tests.

Animal Tests

Inject a mouse intraperitoneally with 0.5 ml. of sputum washed by suspending it in approximately 10 ml. of salt solution and drawing it in and out of a 2-ml. syringe; see fig. 34. If a prompt report is considered essential, make an exploratory puncture after three to six hours and test the peritoneal exudate with type sera. When the type cannot be determined and the mouse is ill, make another puncture the following morning. If the mouse dies, culture the peritoneal exudate and heart's blood and examine films from the exudate; figures 35 and 36. Wash the peritoneal cavity with salt solution in a capillary pipette. Then test the washings with type sera. If the mouse does not die, chloroform after forty-eight hours and autopsy.

If more than one type of pneumococcus is demonstrated, the recovery of any one strain may be facilitated by injecting a mouse intraperitoneally with the sputum and with serum to protect against the other type or types that are present (8).

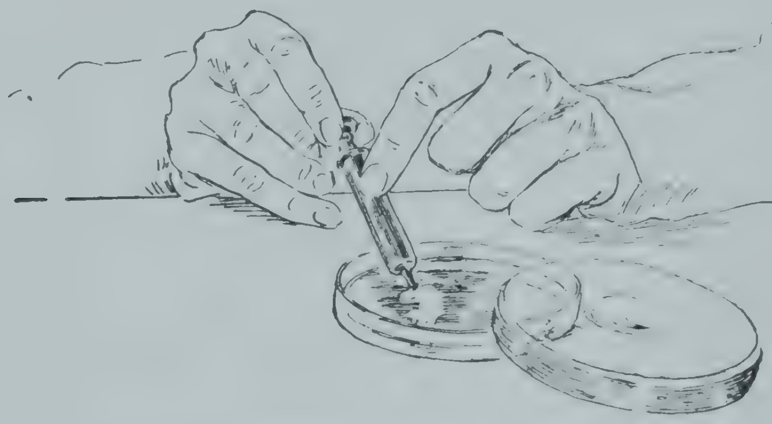

FIG. 34. WASHING SPUTUM IN SALT SOLUTION

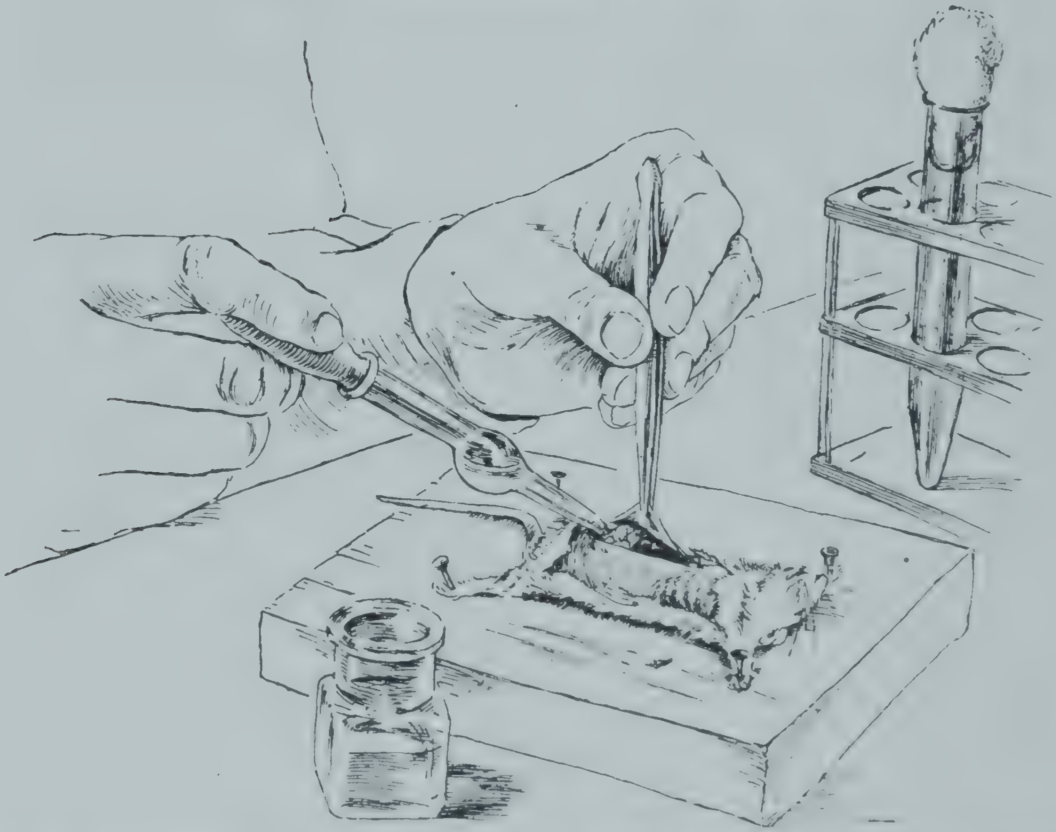

FIG. 35. COLLECTING PERITONEAL WASHINGS FROM A MOUSE PREVIOUSLY INOCULATED WITH SPUTUM

BLOOD

The cultural examination of blood is most satisfactory when undertaken promptly after collection. The use of an appropriate anticoagulant such as sodium citrate is recommended. When a specimen thus collected is received, add a portion to ten to twenty times its volume of semisolid agar containing 1 per cent glucose (*F32D*) that has been warmed to 35°–37°C. If the blood was collected following the administration of a sulfonamide or penicillin, use semisolid medium (*F32D*) to which has been added *p*-aminobenzoic acid or its sodium salt, or penicillin inactivator, respectively. Poured plates should also be prepared to determine the number of pneumococci present unless the specimen has been a day or more in transit. After six or seven hours' incubation, examine films prepared from the semisolid medium. If microorganisms having the morphology of pneumococci are present, test for the *Quellungsreaktion* as described for sputum. If a type is not indicated, attempt identification of the microorganisms by means of cultural, serologic, and if necessary, by animal tests. Should no microorganisms be found, examine again after twenty-four and forty-eight hours.

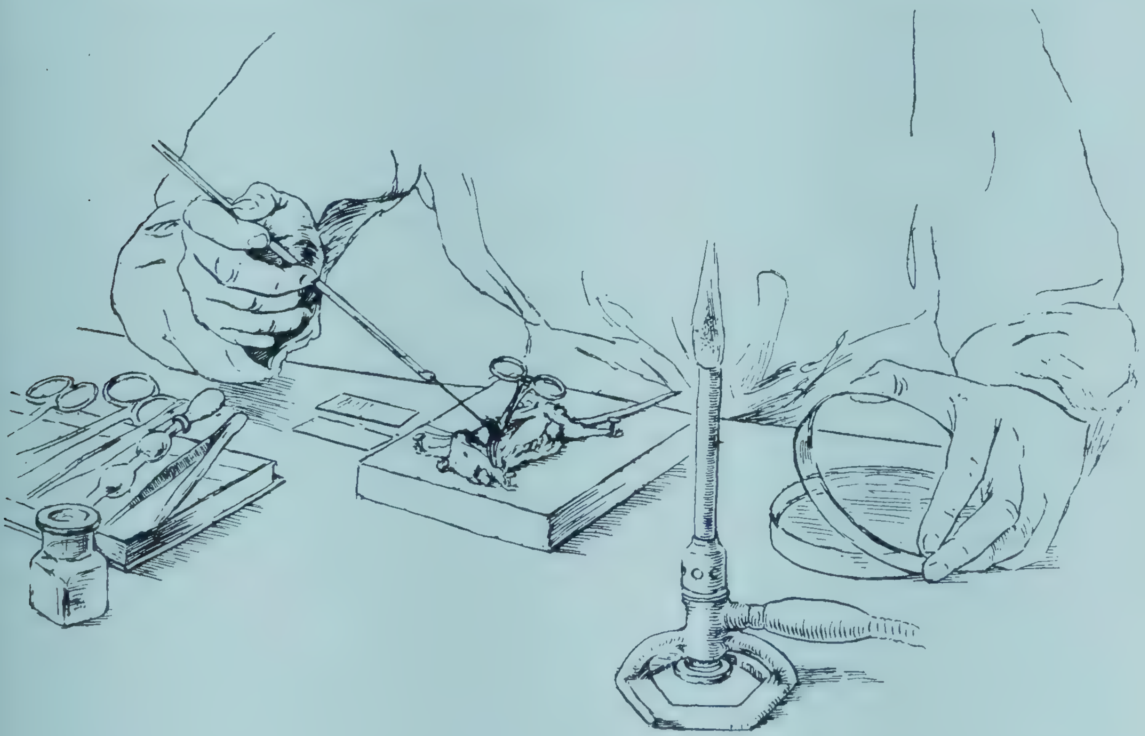

FIG. 36. TAKING BLOOD FROM THE HEART FOR CULTURAL EXAMINATION

When clotted blood or blood in broth is received, proceed as described for cultural examination of blood. (See "General Instructions," p. 263.) Blood cultures from patients with pneumonia should always be examined after eight to twenty-four hours' incubation.

CEREBROSPINAL, PLEURAL, AND OTHER FLUIDS

If Gram-positive cocci in pairs and chains are found in films prepared from the sediment, test with type sera for the *Quellungsreaktion*. If the type cannot be determined or the stained preparation shows no diplococci, make a cultural examination. For cerebrospinal fluid, follow the procedures outlined under "Examination of Cerebrospinal Fluid," p. 517.

STOMACH WASHINGS

When stomach washings are received, determine the reaction. If acid to litmus paper, add sufficient 10-per-cent sodium bicarbonate to neutralize and examine as directed for sputum. Should the specimen consist largely of fluid, centrifuge it and examine the sediment.

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CHAPTER 19

EXAMINATION FOR STREPTOCOCCI AND STAPHYLOCOCCI

HEMOLYTIC STREPTOCOCCI

Hemolytic streptococci are associated with a variety of infections, including scarlet fever, erysipelas, septic sore throat, and puerperal sepsis. Although it is impossible by laboratory procedures to establish a definite etiologic relationship between a specific streptococcus and any of these conditions, the majority of cultures isolated from lesions in man belong to a single serologic group that has been designated A. Thus, the group-precipitation test is especially useful in the study of hemolytic streptococci isolated from cows when outbreaks of streptococcus infection occur among consumers of raw milk from a particular dairy. Information regarding the serologic types of strains, that is whether the cases have been incited by one or more types, is also frequently of importance in the investigation of outbreaks.

NOSE AND THROAT CULTURES

When examinations for hemolytic streptococci cannot be undertaken promptly after collection of the specimen, both a swab and a culture on Loeffler's coagulated-serum medium (*F15*) should be available, since these microorganisms can sometimes be isolated from one and not the other.

Microscopic Examination

Moisten the swab in 2 ml. of beef-extract broth (*F14A*) and examine a slide preparation stained with aqueous alcoholic methylene blue (p. 38). Incubate the culture on coagulated-serum medium for eighteen to twenty-four hours and examine slide preparations stained with methylene blue for diphtheria bacilli as well as streptococci.

Cultural Examination

For the isolation of hemolytic streptococci, use plates of blood agar (*F43A*) containing no added carbohydrate, since this may inhibit hemolytic activity. Incubate one plate in the presence of 5–10 per cent carbon dioxide and another under anaerobic conditions since some strains when freshly isolated grow only in the absence of free oxygen.

Streak both the moist swab and the washings from it on blood agar. After incubating the culture on coagulated-serum medium for eighteen to twenty-four hours, suspend a representative loopful in 2 ml. or more of beef-extract broth and streak blood agar. The degree of dilution will depend upon the number and kind of bacteria in the film. If hemolytic colonies are not found on plates incubated for eighteen to twenty-four hours in the presence of 5–10 per cent carbon dioxide, reincubate and examine on the following day. Examine anaerobic plates after forty-five to forty-eight hours' incubation.

Fish several hemolytic colonies, transferring part of the growth from each to a blood-agar slant and to beef-infusion broth (*F5A*). After incubation, examine a film prepared from the broth culture and stained by Gram's method (p. 35); examine the growth on the blood slant for hemolytic activity. Pneumococci and some strains of staphylococci may produce hemolysis when grown under anaerobic conditions or in the presence of 5–10 per cent carbon dioxide. Also, variants of the recognized serologic groups of hemolytic streptococci may produce hemolysis only under anaerobic conditions.

Corynebacterium pyogenes resembles the hemolytic streptococcus in the production of hemolysis on blood agar. For methods of differentiation, see "Examination for Corynebacteria," p. 287. Colonies of certain strains of the genera *Listerella* and *Hemophilus* may also be mistaken for hemolytic streptococci.

The extent to which procedures for identification and differentiation of hemolytic streptococci are carried is determined by the source of the specimen and the purpose of the examination.

For microorganisms found in pure culture in blood, spinal fluid, and purulent discharge, a study of the morphology in fluid media and of the hemolytic activity on blood agar when incubated under aerobic conditions may furnish all of the information needed. Nevertheless, it is frequently of interest to ascertain the serologic group as determined by the precipitation method of Lancefield (1), especially when other microorganisms are also present in such specimens, or when hemolytic streptococci are isolated from milk or from nose and throat cultures. The strains commonly pathogenic for man produce hemolysis not only when grown on blood agar under aerobic conditions but also when broth cultures are combined with equal amounts of a 5-per-cent suspension of sheep, horse, or human red blood cells (p. 16), and are members of serologic group A.

In special investigations, determination of biochemical (2) and toxigenic (3, 4) properties, and classification according to serologic type (5, 6) yield information of practical value in the study of strains of group A.

Determination of Group (1, 7–11)

Preparation of Extract.—Inoculate 10 ml. of infusion-free medium with thiopeptone (*F21C*)[1] with approximately 0.5 ml. of broth culture or a loopful of growth from a blood-agar slant (*F43A*) and incubate for eighteen to twenty-four hours. Examine a stained preparation and streak a blood-agar plate. If no evidence of contamination is found, separate the bacterial cells by centrifugation. Prepare a 1:20 dilution of *N* hydrochloric acid in salt solution. Mix the cells with 2 ml. of this solution and heat in a boiling water bath for twenty minutes. Cool under running water, centrifuge, and remove the supernatant. To this fluid, add 0.05 ml. of bromothymol blue indicator. Prepare color standards by adding 0.05 ml.

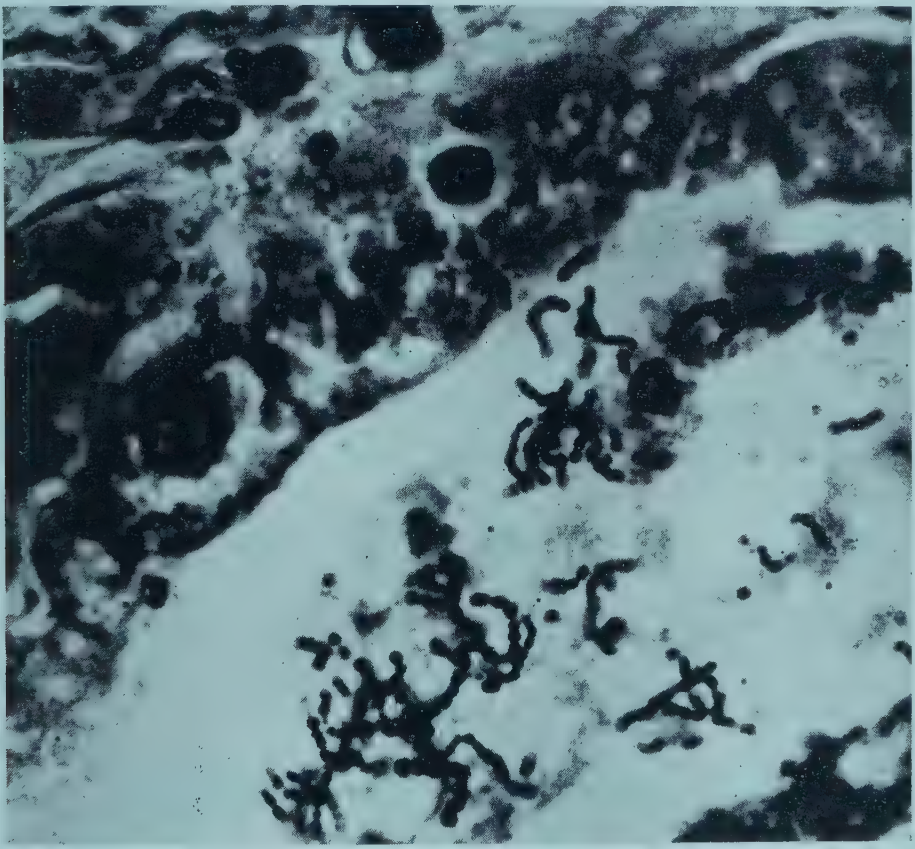

FIG. 37

PHOTOMICROGRAPH SHOWING STREPTOCOCCI IN ALVEOLI OF THE UDDER OF A COW TO WHICH OUTBREAKS OF STREPTOCOCCUS INFECTION WERE TRACED. STREPTOCOCCI OF SEROLOGIC GROUP A WERE ISOLATED FROM THIS TISSUE (× 1300) of the indicator to 2 ml. of the buffer solutions of pH 7.0 and pH 7.2. By comparison with these standards, adjust the fluid to pH 7.0–7.2 with *N* sodium hydroxide. Centrifuge and use the clear supernatant in the test.

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[1] With a few strains, it may be necessary to use beef-infusion broth to obtain sufficient growth. However, care should be taken in the selection of the medium and the preparation of the extract to avoid nonspecific reactions (12) in the test. If evidence of such reactivity is obtained, a new extract should be prepared from cells washed in salt solution.

Precipitation Test. Before sera are used for classification of strains, each is tested for group specificity with extracts of strains of heterologous groups and of different serologic types of the homologous group. Into 8- by 100-mm. tubes pipette 0.1 ml. of undiluted serum (see "General Bacteriologic Technic," p. 21). Add 0.1 ml. of extract on the surface to form a layer. For control, combine similar volumes of each extract and normal rabbit serum, of each serum and salt solution, and of each serum and the extract of a strain of its homologous group. Examine for the presence of a ring precipitate after ten minutes' incubation. Shake the tubes and record the amount of precipitation after two hours' incubation and overnight in the refrigerator.

When the classification according to serologic group has been determined, include with the report of the findings an explanatory note in regard to the usual source of strains belonging to the group concerned.

Determination of Type by Precipitation

Streptococci of serologic group A are typed by the precipitation method (5). Types 10 and 12, which have serologically identical M antigens but different T antigens (13), are differentiated by the slide agglutination method of Griffith (6).

Sera for use in type determination must be of satisfactory potency and free from cross reactivity (see "Sera for the Classification of Streptococci," p. 792).

Preparation of Extract.—Inoculate approximately 500 ml. of infusion-free medium with thiopeptone (*F21C*) with 10–15 ml. of an 18-hour culture in infusion broth (*F5A*). Incubate for eighteen hours. Examine a stained slide preparation and streak a blood-agar plate. If no evidence of contamination is found, centrifuge and discard the supernatant. Dilute *N* hydrochloric acid 1:20 with 0.85-per-cent salt solution. Suspend the packed cells in 3 ml. of this solution. Check the pH by the spot-plate method (14) using 0.04-per-cent thymol blue as indicator. If it is less acid than approximately pH 2.2, adjust to pH 2.2 with *N* HCl. Heat for ten minutes in a boiling water bath, shaking frequently to keep the cells in suspension. Check the pH. If it is less acid than pH 2.2, readjust and heat again for ten minutes. Cool quickly against running water and centrifuge. Decant the supernatant. Determine the pH by the spot-plate method using bromothymol blue indicator, and adjust to pH 7.2 to 7.4 with *N* NaOH. Centrifuge and remove the clear supernatant for use in the test.

For some strains, more satisfactory results may be obtained with extracts prepared as described in "Sera for the Classification of Streptococci," p. 792.

Precipitation Test.—Into 8- by 100-mm. tubes, pipette 0.05 ml. of undiluted, absorbed type specific serum of each of the available types and a group-A antiserum. Add 0.05 ml. of extract on the surface to form a layer. For controls, combine similar volumes of each extract and normal rabbit serum, each serum and salt solution, each serum with an extract of its homologous type. Incubate for ten minutes and record the presence of a ring precipitate. Shake the tubes. Record the results after two hours and after eighteen hours in a refrigerator.

Determination of Type by Agglutination

Preparation of Culture.—From a broth culture or blood-agar slant, inoculate a tube of pig-stomach-digest broth (*F13.1A*) with 0.2-per-cent glucose. Incubate overnight. Streak a blood-agar plate and examine a slide preparation stained by Gram's method (p. 35). If no evidence of contamination is observed, transfer approximately 8 ml. to a 15-ml. centrifuge tube. Centrifuge, discard the greater part of the supernatant, and take up the sediment in a very small amount of the supernatant broth. It may be necessary to make a number of serial transfers in pig-stomach-digest broth before a suspension is obtained that does not agglutinate spontaneously.

Slide-Agglutination Test.—Mark off a glass slide, 5.1 by 7.6 cm., in three sections with a china marking pencil. With a capillary pipette fitted with a rubber bulb, place a drop of cell suspension in each section. To the first drop of the cell suspension, add a loopful of a previously determined dilution of type-10 serum absorbed with type-12 cells, to the second, a loopful of type-12 serum absorbed with type-10 cells, and to the third, normal rabbit serum. The immune serum dilutions are made in normal rabbit serum. Mix with the loop. Tilt the slide several times and read immediately. Set aside and read again after five minutes.

BLOOD CULTURES, CEREBROSPINAL FLUID, AND PURULENT DISCHARGES

Inoculate blood-agar plates (*F43A*) and make film preparations. Isolate and identify the hemolytic streptococci as described for nose and throat cultures.

MILK

Store the milk specimen in the refrigerator until the cream has separated. This usually requires from four to five hours. Stain films from the cream with aqueous alcoholic methylene blue (p. 38) and Newman-Lampert's stain (p. 38), and examine for pus cells and bacteria. Streak the cream on blood agar (*F43A*) and examine as directed for nose and throat cultures.

If the test must be performed immediately, centrifuge the milk at approximately 2000 r.p.m. for one-half hour. With a capillary pipette remove the milk, mix the cream and sediment and examine as described in the preceding paragraph.

STREPTOCOCCI OTHER THAN HEMOLYTIC STREPTOCOCCI

Occasionally streptococci that produce green discoloration on media containing blood, or streptococci that produce neither hemolysis nor green discoloration are isolated from specimens accompanied by history which indicates that these microorganisms may be of pathogenic significance. The procedures for the isolation of these varieties of streptococci are the same as for hemolytic streptococci. Such strains are sometimes members of the serologic groups as determined by the precipitation method (1).

Streptococci producing green discoloration on media containing blood can be differentiated from pneumococci by their failure to dissolve in bile, sodium desoxycholate, or sodium lauryl sulfate (p. 18).

STAPHYLOCOCCI

Staphylococci can usually be easily isolated under aerobic conditions. Occasionally strains have been encountered that will develop only when grown in the presence of 5–10 per cent of carbon dioxide. The cultures should be transferred to coagulated-serum medium (*F15*) in order to determine the degree of pigment formation. When grown at a temperature of 18°–30°C. rather than at approximately 37°C., the color may be more intense.

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CHAPTER 20

EXAMINATION FOR NEISSERIA

NEISSERIA GONORRHOEAE

Although gonococci are demonstrated more frequently by cultural than by microscopic examination, both should, if possible, be undertaken. Not infrequently morphologically typical gonococci may be found in stained preparations when they cannot be isolated. Cultural examination should preferably be undertaken promptly after the collection of the specimen. The results are only slightly less dependable, however, when specimens in satisfactory medium are sent by mail or are otherwise delayed in reaching the laboratory (1, 2).

DISCHARGE

Microscopic Examination

Fix slide preparations by heat and stain by Gram's method (p. 35). Examine discharge from the genital tract or from the eye for Gram-negative intracellular cocci having the morphology of gonococci. The failure to find such microorganisms does not exclude the possibility of gonococcus infection.

When numerous pus cells but no gonococci are found in specimens prepared from the vagina of a child, or from the urethra or cervix, the need for the submission of additional specimens should be stressed. If extracellular Gram-negative cocci are present, this information should also be given in the report.

When no gonococci are found in slide preparations of discharge from eyes, especially in ophthalmia neonatorum, request additional films to be examined for evidence of inclusion blennorrhea. See "Inclusion Blennorrhea," p. 355.

When structures resembling *Trichomonas vaginalis* are found, report this fact. See "*Trichomonas vaginalis*," p. 484.

Cultural Examination

Streak plates of coagulated-blood agar (*F37* or *F55.5A*) with discharge on swabs immediately after collection; within six hours after collection when immersed in semisolid agar (*F32C*); or after longer periods, if in the coagulated-blood-gelatin agar (*F55.5B*) in the mailing outfits, see "Appendix C," p. 886.

Incubate the plates for from forty-four to forty-eight hours in the presence of 5–10 per cent carbon dioxide and preferably at not more than 36°C. Fish small, gray, finely granular colonies to coagulated-blood agar. The oxidase reaction (3, 4) may be helpful in the selection of colonies, especially when large numbers of contaminating bacteria are present. Place a loopful or a drop of an approximate 1-per-cent aqueous solution of dimethyl *p*-phenylene diamine hydrochloride (freshly prepared each day) on or near the colony to be tested. If no colonies typical of gonococci are seen, flood the surface of the plate. Pick immediately any colonies that become pink, since the bactericidal action of the reagent is rapid.

Incubate the fishings for from forty-four to forty-eight hours in the presence of 5–10 per cent carbon dioxide. If Gram-negative cocci are isolated, test for the fermentation of glucose, maltose, sucrose, and lactose in ascitic-fluid semisolid agar (*F39B*) or starch-gelatin agar (*F55.7*). See table 10. Seal the tubes with paraffin. If there is any doubt concerning the identity of the microorganism, inoculate an unenriched medium, such as beef-extract agar (*F49A*), and incubate at 18°–30°C.; gonococci and meningococci will usually not grow under these conditions.

TABLE 10

Fermentation reactions of species of Neisseria

	REACTION IN MEDIA CONTAINING			
	Glucose	Maltose	Sucrose	Lactose
N. gonorrhoeae	Acid	No acid	No acid	No acid
N. meningitidis	Acid	Acid	No acid	No acid
N. catarrhalis	No acid	No acid	No acid	No acid

NEISSERIA MENINGITIDIS

While many species of pathogenic bacteria have been reported as the occasional incitants of meningitis, in cases of purulent meningitis that do not follow an infectious process in the mastoid or elsewhere, the meningococcus (*Neisseria meningitidis*) is the microorganism most frequently encountered. Since meningococci autolyze readily, few may be found in the cerebrospinal fluid, and in some instances, when the examination cannot be made promptly, none can be demonstrated. Experience has indicated that when large numbers of polymorphonuclear leucocytes are present in the cerebrospinal fluid and no bacteria are found, the incitant is usually the meningococcus, which is often found in a later specimen from the patient.

Invasion of the blood stream by *N. meningitidis* may precede or occur independently of meningococcus meningitis. Chronic infection of the blood stream with this microorganism may occur. Cultural examination of a blood specimen collected during a febrile episode, if the fever is intermittent, aids in establishing the diagnosis.

CEREBROSPINAL FLUID

Make a leucocyte count and a colloidal gold test and determine the protein content. (See "Examination of Cerebrospinal Fuid," p. 517.)

Microscopic Examination

Prepare thin films from the sediment and dry rapidly so that the leucocytes will be flattened and intracellular microorganisms will be stained characteristically. Stain by Gram's method (p. 35) and examine for biscuit-shaped, Gram-negative, intracellular cocci that may be tentatively identified as *N. meningitidis.* If only extracellular cocci having the morphology of the genus *Neisseria* are found, report this fact.

Cultural Examination

Streak a blood-agar plate (*F43A*) or a coagulated-blood-agar plate (*F37* or *F55.5A*) with some of the sediment, and incubate in the presence of 5–10 per cent of carbon dioxide. Inoculate, also, ascitic-fluid semisolid or glucose semisolid medium (*F36A* or *F36B*) and incubate for at least four days unless growth occurs sooner. If the fluid was collected following the administration of a sulfonamide, use semisolid medium to which *p*-aminobenzoic acid or its sodium salt has been added. If penicillin has been administered, use semisolid medium to which has been added a penicillin inactivator. Incubate the remaining sediment with about 0.5 ml. of supernatant in the presence of 5–10 per cent of carbon dioxide and plate daily for three days or until growth is obtained. Fish colonies that are gray and glistening and from 1 to 2 mm. in diameter to slants of glucose-serum agar (*F34C*). At times, the growth may be confluent. If Gram-negative cocci are isolated, test for the fermentation of glucose, maltose, sucrose, and lactose in ascitic-fluid semisolid agar (*F39B*) or starch-gelatin agar (*F55.7*). See table 10.

Neisseria gonorrhoeae has occasionally been isolated from cerebrospinal fluid. Although *Neisseria catarrhalis* and chromogenic *Neisseria* have seldom been found under conditions that indicated that they were of pathogenic significance, these species have been reported in specimens of cerebrospinal fluid from patients with meningitis. *Neisseria catarrhalis* forms grayish-white colonies and grows luxuriantly on unenriched media and at room temperature. The growth of certain other species of *Neisseria* is yellowish in color.

Macroscopic Slide-Agglutination Test.—Make this test when colonies suggestive of meningococci are found on the plates. (See "General Bacteriologic Technic," p. 21.) Prepare with salt solution 1:10 dilutions of multivalent antimeningococcus and normal horse sera for this purpose. Stain the preparation by Gram's method (p. 35). Gram-negative cocci agglutinated in antimeningococcus serum only may be tentatively identified as meningococci.

Macroscopic Tube-Agglutination Test.—Make a suspension from the culture grown on blood-agar or serum-glucose-agar slants, and perform an agglutination test with multivalent antimeningococcus and normal horse sera. Incubate for twenty-four hours at 48°–52°C. (See "General Instructions," p. 264.)

In general, meningococci are strongly agglutinated in multivalent antimeningococcus horse serum and not in salt solution or in 1:50 dilution of normal horse serum. However, strains are occasionally isolated that are agglutinated in normal serum and salt solution, or that are agglutinated only slightly, if at all, in the multivalent antimeningococcus serum.

Nonpathogenic Gram-negative cocci that are strongly agglutinated by the normal horse serum, diluted 1:50, are found in the nasopharynx.

If the results of agglutination tests are not satisfactory, transfer the culture daily for a week or ten days and repeat the test, using a wider range of dilutions of the serum. If identification of the serologic group of the culture is desired, follow the directions given on p. 707.

BLOOD

The cultural examination of blood should be undertaken as soon as possible after collection. Inoculate semisolid agar (*F32C*). If the blood was collected following the administration of a sulfonamide, use semisolid medium to which *p*-aminobenzoic acid or its sodium salt has been added. Warm the medium to 35°–37°C. before using and incubate all cultures in the presence of 5–10 per cent of carbon dioxide. Streak the surface of a blood-agar plate (*F43A*) or a coagulated-blood-agar plate (*F37* or *F55.5A*) and examine a stained film from the semisolid medium each day for four days unless growth is obtained earlier. If cocci having the morphology of meningococci are found, identify as directed under "Cerebrospinal Fluid."

NASOPHARYNGEAL SECRETION

Cultural Examination

Directly after collection on a swab, rub the material on a small portion of the surface of a plate of blood agar (*F43A*) and one of coagulated-blood agar (*F37* or *F55.5A*) that have been warmed to approximately 35°–37°C. Streak the surface of the plate with a loop from the portion inoculated with the swab as directed for grossly contaminated material, p. 13, and incubate immediately in the presence of 5–10 per cent carbon dioxide. Whenever the specimen cannot be plated promptly on warmed media and the plates incubated immediately, the examination should be considered unsatisfactory unless *N. meningitidis* is isolated.

Stain films prepared from the swab by Gram's method.

Examine the plates after twenty-four and forty-eight hours' incubation and again after four days. Fish colonies that have the characteristics of those of meningococci and identify them as directed under "Cerebrospinal Fluid," p. 284. *Neisseria catarrhalis* and other Gram-negative micrococci that resemble meningococci in morphology are frequently found in the nasopharynx.

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CHAPTER 21

EXAMINATION FOR CORYNEBACTERIUM

CORYNEBACTERIUM DIPHTHERIAE

The marked decrease in the incidence of diphtheria as a result of immunization has placed a greater responsibility upon the laboratory as a source of information that may aid in diagnosis. The procedure of choice is to isolate and test the virulence of every strain morphologically typical of or suggesting *Corynebacterium diphtheriae.*

DISCHARGE

Incubate immediately cultures on Loeffler's coagulated-serum medium (*F15*) to be examined for *Corynebacterium diphtheriae.* An examination of slide preparations of the cultures stained with aqueous alcoholic methylene blue (p. 38) may be made after ten hours' incubation, and the presence of morphologically typical diphtheria bacilli reported. Unless these microorganisms are found, make a final examination of the culture after eighteen to twenty-four hours' incubation. When very few diphtheria-like bacilli are present, examine additional stained preparations. If the morphology of the bacilli found is atypical, preparations stained by Gram's method (p. 35) or Albert's method (p. 30), may be of assistance. *Corynebacterium diphtheriae* is Gram-positive, and with Albert's stain, is pale green with dark brown or black granules. In all cases, however, base the report on the results obtained with the methylene blue stain. Examine the cultures also for hemolytic streptococci if the clinical data suggest such an examination may be important as an aid in diagnosis. In outbreaks or groups of cases, it may be necessary to identify the particular streptococcus giving rise to the infection.

Inoculate a tube of coagulated-serum medium with the swab returned in the mailing case if (1) a culture on this medium is not received; (2) the culture tube is broken; (3) the swab has been left in the medium; (4) the culture appears contaminated; or (5) more than four days have elapsed since collection of the specimen. After cultures have been incubated for eighteen to twenty-four hours, examine stained preparations.

When films prepared from exudates are received, stain one with aqueous alcoholic methylene blue and one by Gram's method since some Gram-negative bacilli may slightly resemble *C. diphtheriae* when stained with methylene blue. Unless morphologically typical diphtheria bacilli are found, consider the examination unsatisfactory and arrange for the submission of a culture on coagulated-serum medium.

Isolate and test the virulence of diphtheria bacilli or microorganisms resembling them morphologically in first specimens from a given patient. Always attempt to isolate microorganisms the morphology of which suggests, but is not perfectly typical of, diphtheria bacilli, and morphologically typical diphtheria bacilli in cultures from convalescent or contact carriers three months after the date of onset or exposure (1); or in those from noncontact carriers when the history accompanying the specimens indicates the need for this study.

The injection of a suspension of the throat culture intracutaneously in a guinea pig will often furnish data of diagnostic value. The results, however, should be confirmed by study of the microorganism in pure culture (see "Intracutaneous Test, p. 289").

Isolation of Corynebacterium

Suspend a representative loopful of the growth from coagulated-serum medium (*F15*) in ascitic-fluid broth containing potassium tellurite (*F2B*) and inoculate with two or three loopfuls of this suspension, a plate of glucose serum agar containing potassium tellurite with or without copper sulfate (*F40B* or *F40C*). The medium with copper sulfate is particularly useful for the isolation of diphtheria bacilli when the culture contains many staphylococci. Transfer the original culture to coagulated-serum medium. Incubate the inoculated media for eighteen to twenty-four hours. If characteristic colonies are not found, reincubate for an additional day. Also inoculate a second series of plates from the surface growth on the fluid medium.

Colonies of diphtheria bacilli on potassium-tellurite medium are small, colorless, granular, and, when copper sulfate is present, have an irregular outline. Fish such colonies to coagulated-serum medium. When no characteristic colonies can be found on the plates, fish a few of the various types, since the microorganisms seen in the original stained preparations having the morphology of diphtheria bacilli may differ in their colony characteristics.

After the cultures have been incubated, stain slide preparations with aqueous alcoholic methylene blue to determine the morphology and purity. If none of the cultures contains microorganisms that resemble *C. diphtheriae* in morphology or the microorganisms on which the original report was based, examine plates inoculated from the culture in fluid medium. If the microorganisms are not isolated by this method, repeat the procedure with the growth on coagulated-serum medium inoculated with the original culture and include a blood-agar plate (*F43A*).

When microorganisms are isolated that have the morphology of those on which the report of the microscopic examination was based, test the fermentative properties. If these are typical of diphtheria bacilli (see table 11), test for virulence; if not, determine the viability and purity of the cultures in the carbohydrate-serum-water medium and, if necessary, test other fishings. If there is any doubt as to the identity of the culture, test its virulence.

Fermentation Tests

Inoculate one tube each of glucose-, sucrose-, and dextrin-serum-water medium containing an indicator (*F28*). Medium with starch may also be inoculated if the differentiation of groups or types of virulent diphtheria bacilli is considered desirable. Record the reactions after eighteen to twenty hours' and again after forty to forty-eight hours' incubation, as follows:

AC acid and coagulation
A acid
A± very slight acid
— no acid

TABLE 11

Fermentative properties of C. diphtheriae, C. hofmanni, C. xerose, C. ulcerans, and C. pyogenes

	REACTIONS IN MEDIA CONTAINING		
	Glucose	Sucrose	Dextrin
C. diphtheriae	AC	—*	AC
C. hofmanni	—	—	—
C. xerose	AC	AC	—
C. ulcerans	AC	—	AC
C. pyogenes	AC	AC	AC

AC acid and coagulation.
— no change or alkaline.
* Rarely AC.

Tests for Virulence

Intracutaneous Test.—Test for virulence by the intracutaneous method microorganisms that have the fermentative properties of *C. diphtheriae* and any others that suggest this species morphologically. Suspend a 2-mm. loopful of the growth of a pure culture in 2 ml. of veal-infusion broth (*F12*) or beef-extract broth (*F14A*) and adjust the turbidity to that of glass standard No. 1. Inject 0.1 ml. of the suspension intracutaneously in corresponding shaved areas on the sides of two light-colored, preferably white, guinea pigs, weighing approximately 300 grams, one of which has received previously an immunizing dose of about 500 units of diphtheria antitoxin subcutaneously. Test no more than four cultures on one set of animals.

When the microorganisms injected are virulent diphtheria bacilli, redness and slight induration are induced at the end of twenty-four hours at the site of inoculation on the guinea pig unprotected by antitoxin, and a marked necrotic area after forty-eight to seventy-two hours. On the animal protected by antitoxin, no reaction at the site of inoculation other than the injury caused by the needle should be obtained. Should all the cultures tested on one set of animals prove to be nonvirulent, for control, inoculate the guinea pigs with a strain of *C. diphtheriae* known to be virulent.

Record the reaction at the end of twenty-four, forty-eight, and seventy-two hours as follows:

4+ very marked necrosis
3+ necrosis with purple concentric area, edema, and inflammation
2+ purplish discoloration with edema and inflammation
+ brownish discoloration with slight edema and inflammation

Subcutaneous Test.—When the intracutaneous test indicates that a microorganism is not virulent or the results are inconclusive, perform a subcutaneous test. Use a guinea pig weighing approximately 300 grams and inject 2 ml. of a 72-hour veal-infusion broth (*F12*) culture or the growth from an 18- to 20-hour coagulated-serum slant culture suspended in 2 ml. of broth (*F12* or *F14A*). Incubate the tubes containing broth cultures in a slanting position (an angle of about 20°) to provide optimum conditions for toxin production.

Observe the animal daily. If it dies, make a post-mortem examination as soon as possible. Make cultures and stained preparations from the site of inoculation and from the heart's blood. Induration, congestion, and necrosis at the site of inoculation, and congestion of the adrenals are usually found in guinea pigs that die following inoculation with virulent diphtheria bacilli. Pleural fluid may or may not be increased. Diphtheria bacilli should be demonstrated in the stained preparation and in the culture made with the material taken from the site of inoculation.

If the guinea pig is alive and shows no evidence of local or other reaction on the fifth day after the injection, consider the culture nonvirulent and discharge the animal. Suspensions of the growth from coagulated-serum agar may give rise to local lesions only when strains are of low virulence.

When the results are questionable or do not agree with those of the intracutaneous test, repeat the tests using two guinea pigs, one of which has received about 500 units of antitoxin twenty-four hours previously.

CORYNEBACTERIUM ULCERANS (2, 3)

In young cultures (4–8 hour), *Corynebacterium ulcerans* closely resembles *C. diphtheriae* in morphology, some forms being uniformly stained and others having polar bodies. In older cultures, most forms are coccoid. This microorganism may be differentiated from *C. diphtheriae* by the rapid change in morphology, liquefaction of gelatin, and reactions in guinea pigs. In the intracutaneous test (p. 289), this species produces indurated inflammatory reactions on both the unprotected guinea pig and the one that has received diphtheria antitoxin.

CORYNEBACTERIUM PYOGENES

Corynebacterium pyogenes may incite an acute form of mastitis in cows. The microorganism is a small Gram-positive, nonmotile, pleomorphic, irregular-staining rod that sometimes resembles a streptococcus. The methods of isolation from milk correspond to those described for streptococci. On blood agar, the colonies are surrounded by zones of hemolysis and resemble those of hemolytic streptococci. However, they may readily be differentiated on coagulated-serum medium and in broth. The serum medium is pitted by *C. pyogenes* but not by streptococci and the latter form chains in broth.

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CHAPTER 22

EXAMINATION FOR MYCOBACTERIUM

The aid in diagnosis which the laboratory can furnish by demonstrating tubercle bacilli in sputum or other types of specimens is too generally recognized to require emphasis. Tubercle bacilli, however, will not be present in specimens unless the tuberculous process has progressed sufficiently to yield necrotic material that contains the bacteria. Thus, in pulmonary tuberculosis, evidence of the disease can usually be demonstrated by means of X-ray and clinical manifestations before tubercle bacilli can be found.

The complement-fixation test is sometimes of value in aiding in the differential diagnosis of active tuberculosis. The reaction occurs in a high percentage of active pulmonary infections and in a smaller percentage of extrapulmonary forms of the disease. The infrequent and low-grade reactions obtained in inactive cases indicate that the reaction of the serum diminishes or disappears with healing of the lesions and apparent arrest of the disease (1–3).

Reactions with tubercle antigens have been reported to occur in the sera of persons suffering from syphilis, malaria, and leprosy. In the sera of patients with leprosy (4), reactions are frequently obtained and are usually marked in degree. In fact, the proportion of marked reactions is greater than is commonly observed in patients with tuberculosis. In syphilis, while reactions occasionally occur, they usually are of low degree. Serologic tests for syphilis are performed, as a control, on all specimens submitted for the complement-fixation test for tuberculosis.

MYCOBACTERIUM TUBERCULOSIS

Microsçopic Examination

Prepare films on new slides, directly or after concentration, or, if the specimen is a watery fluid, from sediment after centrifuging for one-half hour at 2000 r.p.m. or for ten minutes at 3000 r.p.m. Use only reagents filtered through diatomaceous candles or those prepared with freshly distilled or filtered water to exclude saprophytic acid-fast bacilli. Stain by the Ziehl-Neelsen method or a satisfactory modification (p. 43).

Alkali Concentration (5).—Combine the specimen or sediment after centrifuging with an equal amount of 3-per-cent (0.75 *N*) or 4-per-cent (*N*) sodium hydroxide, shake in a shaking machine for ten minutes, and incubate for from thirty minutes to one hour. If the specimen is not completely homogenized, repeat the shaking and incubation or add more sodium hydroxide, providing the entire procedure can be completed within two hours.

Centrifuge at 2000 r.p.m. for thirty minutes or at 3000 r.p.m. for ten minutes. Discard the supernatant in a pail containing undiluted cresol compound. Adjust the pH of the sediment to 6.4–6.6 by adding phosphate buffer solution (6) with bromothymol blue indicator (*F63.7*) until a green color appears. Centrifuge again and discard the supernatant, as previously directed.

Cultural Examination

Tubercle bacilli can be demonstrated by cultural examination in an appreciable percentage of specimens when present in such small numbers that they cannot be found microscopically (7).

When the material is fluid and not contaminated, centrifuge and use the untreated sediment. Otherwise, treat with alkali the specimen or the sediment obtained by centrifuging.

Inoculate with the sediment three or four slants of egg media (*F17*, *F17.2* or satisfactory modifications) in tubes or jars with screw caps, paraffin-sealed cotton plugs, or both cotton plugs and corks or rubber stoppers. Prick the paraffin-sealed cotton plugs once with a needle to permit a very slow interchange of air. Incubate the slants in a horizontal position for twenty-four hours and then in a vertical position.

One medium should contain no glycerol and the other from 4 to 5-percent glycerol to provide optimum conditions for bovine and human types of tubercle bacilli, respectively. Examine the cultures at the end of two weeks and once each week thereafter for eight weeks or until growth characteristic of tubercle bacilli is observed. Report the isolation of acid-fast bacilli having the morphology and growth characters of tubercle bacilli. If the identity of the acid-fast bacilli found is questionable, test for growth on beef-infusion agar and, if necessary, inject a guinea pig.

Animal Tests

The demonstration of acid-fast bacilli in typical lesions in guinea pigs provides the final criterion for the identification of tubercle bacilli. The animals should be inoculated when the source of the specimen or the character of the growth obtained in cultures leaves any doubt as to the identity of the acid-fast bacilli and when these microorganisms may be present in extremely small numbers as in urine collected from the ureter, joint fluid, ascitic fluid, and pus. The inoculation of an animal with a specimen of cerebrospinal fluid is seldom required since data could not be made available early enough to assist in the management of the case.

Prepare specimens for animal inoculation as directed for cultural examination. Suspend the sediment or culture in 1–2 ml. of salt solution and inject subcutaneously into the groin of a guinea pig.

Inspect the animals carefully at least once a week and isolate those that show enlarged glands, skin lesions, or any evidence of illness. In the case of discharging glands, examine stained preparations for acid-fast bacilli and, if they are found, kill the animal and autopsy at once.

Autopsy all animals that die. At the end of eight weeks, kill and autopsy those that remain. Examine the site of inoculation and remove any glands that show nodules, abscesses, or enlargement. Then examine the viscera for tuberculous lesions. If no abnormalities are found, report without making further tests. If definite or suggestive lesions are observed, remove a part of each abnormal organ and examine stained preparations for acid-fast bacilli. If none is found, make cultures to determine whether a secondary infection has occurred. If tuberculous lesions are present but the lymph nodes that receive drainage from the site of inoculation are not involved, an infection from some source other than the specimen is suggested. When the results of the examination of gross material and stained preparations are inconclusive, have a histologic examination made of the liver, spleen, lungs, and lymph nodes.

SPUTUM

Record the amount and character of the sputum. Make a microscopic examination. Unless acid-fast bacilli are found, consider the specimen unsatisfactory if it has the appearance of saliva. Do not culture this type of specimen.

Report the presence of acid-fast bacilli when eight or more having the morphology of tubercle bacilli are found. Examine a second stained preparation when the results of the examination of the first are not entirely satisfactory. Culture the sediment when no acid-fast bacilli or less than eight are found or the morphology is not perfectly typical of tubercle bacilli.

GASTRIC LAVAGE

The examination of gastric lavage often aids in diagnosis and in the evaluation of treatment of patients thought or known to have pulmonary tuberculosis when sufficient sputum cannot be obtained for satisfactory examination or tubercle bacilli cannot be demonstrated in sputum (8–11).

Centrifuge at 2000 r.p.m. for thirty minutes or at 3000 r.p.m. for ten minutes and discard all but approximately 10 ml. of the supernatant. Treat with alkali and make a cultural examination.

CEREBROSPINAL FLUID

If a coagulum or fibrin web is present, transfer it to a slide and spread with a needle. Then make a microscopic examination. Make a prolonged search for tubercle bacilli, at least thirty minutes with a mechanical stage unless they are found sooner. The bacilli may be present in such small numbers that they can be detected only by means of animal inoculation or cultural examination. The findings under such conditions, however, have seldom been available in time to be helpful as an aid in therapy.

Examine also as directed in "Examination of Cerebrospinal Fluid," p. 517.

URINE

When renal tuberculosis is suspected, specimens from both ureters should be examined in order that valuable time will not be lost. Make a prolonged search of stained preparations for acid-fast bacilli which, in specimens properly collected with a catheter, are almost always tubercle bacilli. Regardless of the findings, inoculate a guinea pig and if there is sufficient sediment, make a cultural examination. Specimens from the bladder found to contain acid-fast bacilli require animal inoculation for identification of the microorganism.

BLOOD

For serologic examination, see complement-fixation test for tuberculosis, p. 361.

MYCOBACTERIUM LEPRAE

The clinical data, the source of the specimen, and the result of the microscopic examination may be sufficient for differentiating *Mycobacterium leprae* from *Mycobacterium tuberculosis* and other acid-fast bacilli. Cultural examination and guinea pig inoculation may, however, be necessary.

Microscopic Examination

Examine films from swabbings from the nasal mucosa or from crushed nodular lesions by the Ziehl-Neelsen method or a satisfactory modification (p. 43). Histologic sections prepared from fixed tissue obtained at biopsy can be stained in accordance with the method for demonstrating tubercle bacilli. The presence of large numbers of acid-fast bacilli is usually significant, especially if many of these are packed in the mononuclear cells.

Animal Tests

When the diagnosis is doubtful, inject some of the material into guinea pigs. *Mycobacterium leprae* is nonpathogenic for these animals whereas *Myco. tuberculosis* induces characteristic lesions.

Serologic Tests

The blood from patients with leprosy usually reacts to a marked degree in complement-fixation tests with the tubercle antigen (4). However, in complement-fixation tests for syphilis with the antigens and technic described (p. 361), reactions of marked degree are relatively infrequent. The findings in these serologic tests may thus offer some aid in diagnosis.

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CHAPTER 23

EXAMINATION FOR EVIDENCE OF INFECTION WITH BACTERIAL INCITANTS OF ENTERIC DISEASE

The isolation of the inciting microorganism is of primary importance as an aid in diagnosis and in the detection of carriers of bacillary incitants of enteric disease. Work in this field has been materially advanced by the use of selective enrichment and plating media that are designed to permit the growth of the pathogenic forms and inhibit certain of the nonpathogenic species that otherwise would tend to overgrow the former microorganisms that are present. Knowledge of the bacillary incitants of enteric disease has increased materially in the past few years and the number of the species of pathogens in this general group is undoubtedly still far from complete. Hence, it is important to study fully the microorganisms found in specimens of stools.

Agglutination tests with patient's serum may furnish preliminary findings of material value in the case of typhoid fever, but in salmonella infections they are seldom helpful as an aid in diagnosis because of the diversity of the antigenic structure of this species. With dysentery bacilli, the test does not provide sufficient aid in diagnosis to warrant undertaking the work, at least as a routine procedure.

BACTERIUM TYPHOSUM, BACTERIUM DYSENTERIAE, AND SALMONELLA

Specimens of feces, urine, blood, duodenal drainage, and gall bladders are examined for *Bacterium typhosum* and *Salmonella*. Specimens of feces are also examined for dysentery bacilli, unless contraindicated by clinical, epidemiologic, or laboratory findings. (See table 12.)

FECES

Consider feces satisfactory for examination only when the specimen is received (1) within four days after collection in approximately ten times its volume of a satisfactorily standardized preservative, such as buffered 30-per-cent glycerol solution (*F70*), or (2) within a few hours after collection if no preservative is used. When a specimen is received without preservative, emulsify approximately 1 gram in 10–15 ml. of buffered 30-per-cent glycerol solution.

Isolation and Identification of Microorganism

Employ plating media dispensed in Petri plates with porous earthenware covers. Store bismuth sulfite agar in the refrigerator; and Endo, eosin methylene blue, and citrate agar at room temperature protected from light, or in the refrigerator. Do not use more than five days after preparation. With an elongated loop (5 by 12 mm.) bent at an angle of approximately 30°, place one loopful of the upper layer of the fecal suspension on one plate of Endo agar (*F52A*) or eosin methylene blue agar (*F52.4*) and two to four loopfuls on one plate each of citrate agar (*F44* or *F50.5*) and bismuth sulfite agar (*F51*). Streak in parallel rows approximately 2 mm. apart, touching the inoculum with the loop only once. For comparison and control, inoculate sections of plates of each kind of medium with pure cultures of *Bact. typhosum*, *Salmonella*, dysentery bacilli, and *Bacterium coli*. Use sufficiently diluted suspensions of cultures to ensure the development of isolated colonies. Specimens of feces known to contain bacillary incitants of enteric disease may also be plated as an additional control for the media.

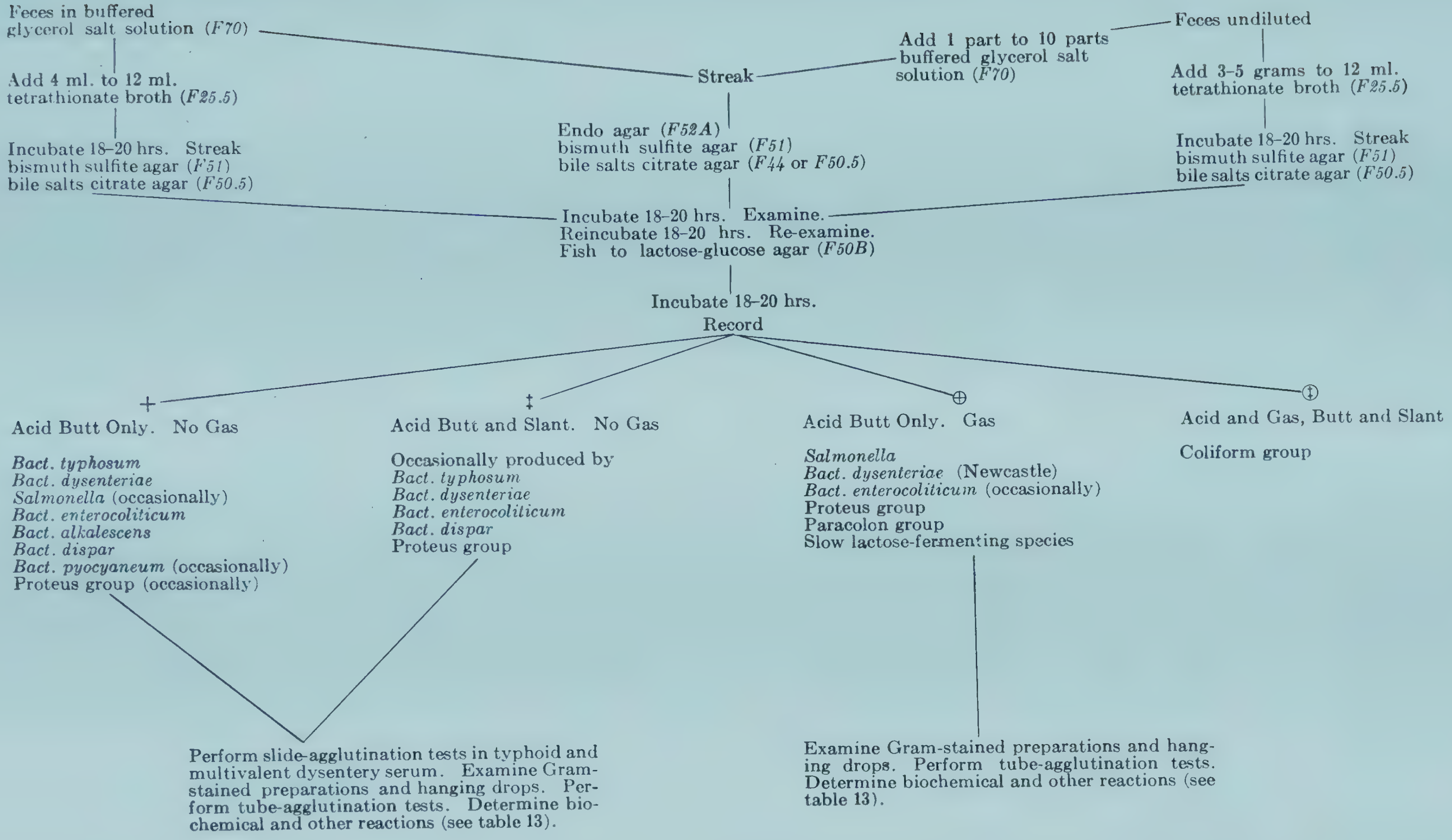
To facilitate the isolation of *Bact. typhosum* and *Salmonella*, add approximately 4 ml. of fecal suspension in glycerol solution or 3–5 grams of undiluted feces to 12 ml. of peptone tetrathionate medium (*F25.5*), to which 0.3 ml. of iodine solution has been added immediately preceding use. After incubation for from eighteen to twenty hours, streak the tetrathionate broth enrichment culture on one plate each of bismuth sulfite agar and bile salts citrate agar (*F50.5*).

The addition of formalin to give final concentrations of 1:7500 and 1:10,000 respectively to two portions of the stool according to the method recommended by Kligler, Olcinik, and Czazkes (1) may aid in the isolation of dysentery bacilli.

After incubation for from eighteen to twenty hours, examine the plates with a microscope or hand lens. Reincubate bismuth sulfite agar and citrate agar and re-examine after from eighteen to twenty hours unless microorganisms of diagnostic significance have already been isolated. *Bacterium typhosum*, *Salmonella*, and dysentery bacilli produce translucent, grayish, or colorless colonies on Endo and eosin methylene blue agar and colorless or delicate pink colonies on citrate agar. In from eighteen to twenty hours on bismuth sulfite agar, unless this is too heavily seeded, *Bact. typhosum* and *Salmonella* usually produce black or brown colonies surrounded by the characteristic halo of darkened medium. However, only light green or colorless colonies may develop which generally become dark and exhibit a halo after further incubation for from eighteen to twenty hours.

TABLE 12

Procedures in the examination of feces



Feces in buffered glycerol salt solution (*F70*)

Add 4 ml. to 12 ml. tetrathionate broth (*F25.5*)

Incubate 18–20 hrs. Streak bismuth sulfite agar (*F51*) bile salts citrate agar (*F50.5*)

Feces undiluted

Add 1 part to 10 parts buffered glycerol salt solution (*F70*)

Add 3–5 grams to 12 ml. tetrathionate broth (*F25.5*)

Incubate 18–20 hrs. Streak bismuth sulfite agar (*F51*) bile salts citrate agar (*F50.5*)

Streak

Endo agar (*F52A*)
bismuth sulfite agar (*F51*)
bile salts citrate agar (*F44* or *F50.5*)

Incubate 18–20 hrs. Examine.
Reincubate 18–20 hrs. Re-examine.
Fish to lactose-glucose agar (*F50B*)

Incubate 18–20 hrs.
Record

+
Acid Butt Only. No Gas

Bact. typhosum
Bact. dysenteriae
Salmonella (occasionally)
Bact. enterocoliticum
Bact. alkalescens
Bact. dispar
Bact. pyocyaneum (occasionally)
Proteus group (occasionally)

‡
Acid Butt and Slant. No Gas

Occasionally produced by
Bact. typhosum
Bact. dysenteriae
Bact. enterocoliticum
Bact. dispar
Proteus group

Perform slide-agglutination tests in typhoid and multivalent dysentery serum. Examine Gram-stained preparations and hanging drops. Perform tube-agglutination tests. Determine biochemical and other reactions (see table 13).

⊕
Acid Butt Only. Gas

Salmonella
Bact. dysenteriae (Newcastle)
Bact. enterocoliticum (occasionally)
Proteus group
Paracolon group
Slow lactose-fermenting species

Examine Gram-stained preparations and hanging drops. Perform tube-agglutination tests. Determine biochemical and other reactions (see table 13).

(‡)
Acid and Gas, Butt and Slant

Coliform group

TABLE 13

Criteria for identification of bacteria inciting enteric disease and of other Gram-negative bacilli often found in feces

SPECIES	REACTION IN LACTOSE-GLUCOSE AGAR (*F50B*)	MOTILITY	REACTIONS IN MEDIA CONTAINING								INDOLE	MILK	GELATIN	SEROLOGIC REACTIONS
			Glucose	Maltose	Mannitol	Lactose	Sucrose	Xylose	Rhamnose	Dulcitol				
Bact. typhosum	+	Motile*	A	A	A	–	–	V	–	– or (A±)	I–	A± or Alk±	NL	Agglutination in *Bact. typhosum* serum
Bact. dysenteriae														
(Shiga)	+	Nonmotile	A	– or A±	–	–	–	–	–	–	I–	A± or Alk±	NL	Agglutination in multivalent (Flexner-Shiga) and/or univalent sera for members of this group
(Schmitz)........	+	Nonmotile	A	V	–	–	–	–	A	–	I+	A± or Alk±	NL	
(Sonne)..........	+	Nonmotile	A	A or A±	A	A±	A±	– or (A±)	A or A±	–	I–	A or AC	NL	
(Flexner)	+	Nonmotile	A	V	A	–	– or A±	–	– or A±	– or A±	V	A± or Alk±	NL	
(Boyd) (2).......	+	Nonmotile	A	– or A±	A	–	–	– or A±	–	– or A±	V	A± or Alk±	NL	
(Newcastle) (3-8)	+ or ⊕	Nonmotile	A	– or A±	– or A	–	–	– or A±	–	– or A±	I–	A± or Alk±	NL	
Bact. dispar........	+	Nonmotile	A	A	A	A±	V	V	V	V	I+	A or AC	NL	Of questionable value
Bact. alkalescens.	+	Nonmotile	A	A	A	–	–	A	A	A or A±	I+	A to Alk	NL	Agglutination in *Bact. alkalescens* and multivalent (Flexner - Shiga) dysentery serum
Bact. enterocoliticum (9, 10), see p. 328	V	Motile†	AG	AG	AG	AG±	AG	AG	–	–	I+	A± or Alk±	NL	Agglutination in *Bact. enterocoliticum* serum
Salmonella........	⊕‡	Motile*	AG	AG	AG	–	–	AG or (–)	AG or (–)	AG or –	I– or (I+)	A± or A to Alk	NL	Agglutination in multivalent and/or univalent sera for members of this group
S. paratyphi A...	⊕‡	Motile*	AG	AG	AG	–	–	–	AG	AG	I–	A±	NL	Agglutination in salmonella 0–II serum
S. paratyphi B..	⊕‡	Motile*	AG	AG	AG	–	–	AG	AG or (–)	AG or (–)	I–	A or A to Alk	NL	Agglutination in salmonella 0 IV,V, H b, and H 1, 2... sera§
Proteus	⊕ or +	Motile*	AG or A	V	V	–	V	V	V	V	V	V	L or L±	Of questionable value

Proteus morganii...	⊕ or +	Motile*	AG	—	—	—	V	—	—	—	I+	— or Alk	NL	Of questionable value
Bact. faecalis-alcaligenes..........	—	Motile*	—	—	—	—	—	—	—	—	I—	Alk	NL	Unknown
Bact. pyocyaneum..	— or +‖	Motile*	— or A	—	—	—	—	—	—	—	I—	Alk to CP	L	Unknown
Paracolon group (11, 12).........	⊕	Motile*	AG	AG	AG	— or AG±	— or AG±	AG	AG or (—)	V	I+ or (I—)	A± or Alk±	NL or (L±)	Various types may be differentiated
Coliform group.....	‡⃝ (⊕)	V	AG	V	AG	AG	V	V	V	V	V	A or AC	V	Of questionable value

* Nonmotile strains sometimes found, especially when freshly isolated.
† Usually motile only when grown at temperatures of 18°–30°C.
‡ Rarely no gas in media containing carbohydrates.
§ Failure to agglutinate in H-b and/or H-1, 2... sera does not disprove *S. paratyphi*-B but agglutination in both is necessary for identification.
‖ Rarely produces gas.
+ = Acid butt, neutral or alkaline slant, no gas.
⊕ = Acid and gas in butt, neutral or alkaline slant.
‡⃝ = Acid and gas throughout.

A = Acid.
G = Gas.
Alk = Alkaline.
C = Coagulated.
P = Peptonized.
± = Weak or delayed reaction.
— = No perceptible change.
( ) = Rare occurrence.

I+ = Indole produced.
I— = No indole produced.
L = Liquefied.
NL = Not liquefied.
V = Variable.

Bacterium coli and related species usually produce opaque colonies which, on Endo medium, may be red, with a metallic luster; and on eosin methylene blue agar, grayish with a blue or black center. Most of these species are markedly inhibited on citrate and bismuth sulfite agar but some may form deep-pink, raised colonies on the former and brown colonies on the latter.

Fish typical or suggestive colonies to lactose-glucose agar (*F50B*), making a deep puncture into the butt and then carrying the needle along the surface of the slant.

If the patient has either an acute or chronic form of enteritis, study also the predominating type of bacteria other than those belonging to types found normally in the intestinal contents of persons who are not ill.

After from eighteen to twenty hours' incubation, record the number of cultures fished from each kind of medium and the reactions obtained in the lactose-glucose agar, using the symbols explained in table 13. Test in appropriate sera for macroscopic-slide agglutination when this may aid in the identification of the microorganism. Stain slide preparations by Gram's method (p. 35), and examine for purity and morphology. Transfer the cultures to beef-infusion (*F2A*) or extract broth (*F14A*) and after two hours' incubation, examine a hanging drop for motility. If gas production is questionable in lactose-glucose agar, inoculate glucose broth (*F14B*) in a double fermentation tube.

Agglutination Test.—Perform a macroscopic tube-agglutination test with the appropriate sera (see "General Instructions," p. 264). Use a 2- to 4-hour broth culture or a suspension of an 18- to 20-hour agar-slant culture in salt solution. If no agglutination is obtained and other properties are characteristic of a recognized species, repeat the test at 48°–52°C. for from fifteen to twenty hours; incubate a second test at 35°–37°C. for two hours and leave in a refrigerator overnight before recording the reaction (see table 13).

When a Gram-negative bacillus is isolated that has the properties of one of the bacillary incitants of enteric disease but is not agglutinated, maintain a broth or agar culture at room temperature from one to several days and test a transfer for agglutination in appropriate serum. If the motility of the microorganisms is questionable, examine a culture maintained at room temperature and make a flagella stain (p. 33). Agglutination tests with the patient's serum may be of value.

Criteria for identification are summarized in table 13. *Bacterium typhosum* can usually be identified by morphology, reaction in lactose-glucose agar, and tube agglutination in a specific dilution of *Bact. typhosum* agglutinating serum. Many or all of the properties described may need to be studied to identify other species. When the microorganism indicated has been isolated from a previous specimen from the same patient or from patients concerned in the same outbreak of enteric disease, the identification can be based on morphology, reaction in lactose-glucose agar, and slide agglutination.

Typing of Bacterium typhosum with Bacteriophage

The differentiation of types of *Bact. typhosum* with bacteriophage as outlined by Craigie provides information that is often helpful to the epidemiologist in showing whether or not the strains isolated from the patients are of the same type as the one found in specimens from the suspected carrier. The preparations have been supplied by Dr. James Craigie, University of Toronto, and more recently by Dr. J. M. Desranleau, Ministry of Health, Montreal. The technic recommended by them is employed (13–16).

Maintain the standard strains on inspissated egg medium (*F15.6*). Inoculate each slant with one streak from the base to the tip. Incubate for from sixteen to eighteen hours, seal with paraffin, and store in the refrigerator. Keep one culture of each type unopened for transplants at the end of three months. Avoid subjecting to room temperature for any longer than is absolutely necessary these or any other cultures in which it is important to preserve the Vi antigen. Transplant freshly isolated strains of *Bact. typhosum* on beef-extract agar pH 6.0 (*F49.5A*) to prevent loss of sensitivity to bacteriophage and to inhibit symbiotic bacteriophage that may be present in the culture. After incubation for from eighteen to twenty hours, store in a refrigerator. Dilute the bacteriophage preparations with beef-extract broth (*F14A*) pH 7.8. Employ the critical test concentration (CTC) recommended by the laboratory supplying them.

Technic of the Test.—On the day that the tests are to be performed, inoculate very lightly beef-extract broth (*F14A*) with the cultures to be tested, including standard strains for control. Incubate until growth is just perceptible. Refrigerate the broth cultures, if they cannot be tested at once. Test for sensitivity to bacteriophage on beef-extract glycerol agar pH 7.8 (*F49.5B*) in Petri plates. The agar should be of uniform thickness, dried to an optimum degree at 35°–37°C. and then stored in a refrigerator until required.

Use platinum wire, No. 20B and S gauge, loops with inside diameter 2.75 mm., to transfer the broth culture and bacteriophage to the agar. Mount each loop in a long handle and bend at an angle to the wire shank so that it will lie parallel with the agar surface during inoculation. Divide the plate into from six to nine sections by marking the bottom with a wax pencil or glass marking ink. Lower a loopful of culture until the drop touches the surface of the agar and spread over an area approximately

15 mm. in diameter with a slight horizontal circular movement. Inoculate a number of areas corresponding to the number of bacteriophage preparations to be used in the test. When the fluid has been absorbed by the agar (5–10 min.), apply a loopful of bacteriophage preparation to the center of the inoculated area. As soon as the fluid has dried sufficiently so that the plates may be safely moved, place them in a refrigerator for two hours and then incubate overnight. Record the reactions as follows:

CL confluent lysis
SCP semiconfluent plaques
3+, 2+, + many, moderate number, and few isolated plaques, respectively
L, N, S, M large, normal, small, and minute plaques respectively

Test each culture first with bacteriophage types I, III, IV, pool B (B_2, B_3, and B_4), C, pool D (D_1, D_2, D_3, and D_4), E (E_1 and E_2), F (F_1 and F_2), and pool of G, J, L, and M. When lysis is obtained in one of the pools, test with appropriate individual bacteriophage preparations to determine the type. No distinction is made between E_1 and E_2, F_1 and F_2, and G and H. For purposes of control, test bacteriophage types I, III, and IV, and pools with a standard type A strain (sensitive to all) and the univalent preparations with standard strains of the homologous types every time that tests are performed.

Test each strain at least twice before reporting the type.

Identification of Salmonella

The Salmonella Subcommittee of the International Association of Microbiologists (17, 18) has defined the genus *Salmonella* as follows: "A large genus of serologically related, Gram-negative and nonsporing bacilli; . . . adhering to the pattern of *B. typhosus* in staining properties and morphology. Failing to ferment sucrose or to clot milk and rarely fermenting lactose, liquefying gelatin or producing indole, they regularly attack glucose with, but occasionally without, gas production. All the known species are pathogenic for man, animals or both."

The identification of *Salmonella* according to the Kauffmann-White classification (17–20) is based on the antigenic structure. More than thirty somatic or 0 antigens have been identified and are denoted by Roman numerals I, II, III, etc. Most species possess two or more somatic antigens and they are divided into groups on the basis of their antigenic structure:

GROUP	SOMATIC ANTIGENS POSSESSED BY ALL SPECIES IN THE GROUP	OTHER SOMATIC ANTIGENS THAT MAY BE PRESENT	TYPE SPECIES
A	II.XII	I	*S. paratyphi*-A. This is the only species in this group at present
B	IV.XII	I.V.XXVII	*S. paratyphi*-B
C	VI	VII.VIII	*S. paratyphi*-C
D	IX.XII	I	*S. enteritidis*
E	III	I.X.XV.XIX.XXVI	*S. london*
Others		Various combinations of these and others	

The majority of *Salmonella* species possess two or more flagellar or H antigens significant in their identification and these are denoted by small letters, a, b, c, etc., and by Arabic numerals 1, 2, 3, etc. These often occur in groups of two or three; either all or none of a given group are present, depending on the phase of the strain. In phase 1, a strain possesses only antigens designated by letters, while in phase 2, either antigens of the type designated by letter or by Arabic numeral but not both may be present. When the antigens of both phases are present, the strain is said to be in the mixed phase. Some species are monophasic.

An International Salmonella Center was maintained in the State Serum Institute, Copenhagen, from which standard strains of all salmonella species and sera for their identification were distributed previous to 1940. The Division of Laboratories and Research received these sera as one of the three salmonella centers in the United States. The supply of sera is sufficient for slide-agglutination tests only and therefore many species cannot be completely identified. Multivalent salmonella serum and univalent sera for the identification of *S. paratyphi*-A and *S. paratyphi*-B are produced in this laboratory (see: "Preparation of sera for identification of Salmonella," p. 787). All but a few of the relatively rare species are agglutinated in the multivalent serum. The univalent sera are designated as 0-II, 0-IV.V, H-b, H-1, 2 . . . to indicate the antigenic factors with which they react (21).

When a microorganism is isolated that has the morphologic, cultural, and biochemical properties of *Salmonella*, perform a tube-agglutination test in multivalent salmonella serum and test the biochemical properties listed in table 13. If agglutination is obtained and the other properties are those of *Salmonella*, perform tube-agglutination tests with salmonella sera designated as 0-II, 0-IV.V, H-b, and H-1, 2 . . . . (See table 14.)

When a strain cannot be identified as *S. paratyphi*-A or *S. paratyphi*-B, perform slide-agglutination tests in sera supplied by the International

Salmonella Center. Suspend in 0.6 ml. of 0.2-per-cent phenol salt solution, the growth from two slants of meat-infusion agar (*F33*) in 13- by 125-mm. tubes. First test cultures grown at room temperature and if the strain cannot be identified, repeat with those grown at 35°–37°C. Combine on a glass slide, one loopful of suspension with one loopful of each serum diluted with 0.2-per-cent phenol salt solution. Tilt the slide back and forth several times and record

4+ prompt, complete clumping
3+ prompt, definite clumping
2+ slow, definite clumping
+ slow, partial clumping
– no clumping

TABLE 14

Identification of S. paratyphi-A and S. paratyphi-B

AGGLUTINATION OBTAINED IN SERUM				SPECIES
0–II	0–IV.V	H–b	H–1, 2 . . .	
Agg.	–	–	–	*S. paratyphi*-A
–	Agg.	Agg.	Agg.	*S. paratyphi*-B
–	Agg.	–	–	Salmonella species of group B; may be nonmotile strain of *S. paratyphi*-B
–	Agg.	Agg.	–	Salmonella species of group B; may be *S. paratyphi*-B in phase 1 or phase 2
–	Agg.	–	Agg.	
–	–	Agg. or –	Agg. or –	Salmonella species other than *S. paratyphi*-A or *S. paratyphi*-B

URINE

Consider urine satisfactory for examination only when it is received within four days after collection. Streak two 12- by 5-mm. loopfuls on a plate of Endo (*F52A*) and on one of bismuth sulfite agar (*F51*). Pipette 2–4 ml. into 12 ml. of peptone tetrathionate medium (*F25.5*), to which the 0.3 ml. of iodine solution has been added immediately preceding use, incubate for from eighteen to twenty hours, and streak one plate each of bismuth sulfite agar and bile salts citrate agar (*F50.5*).

Examine as directed for feces.

DUODENAL DRAINAGE

The appearance of specimens of duodenal drainage should indicate the presence of bile, and the reaction to litmus paper should be alkaline, neutral, or only very slightly acid (22). Otherwise, consider the specimen unsatisfactory unless a microorganism of diagnostic significance is isolated.

Stain a slide preparation by Gram's method (p. 35). Streak the specimen on plates of Endo (*F52A*) and bismuth sulfite agar (*F51*) and pipette 1–2 ml. into (a) 12 ml. of peptone tetrathionate medium (*F25.5*), to which the 0.3 ml. of iodine solution has been added immediately preceding use, and (b) 6–8 ml. of beef-extract broth (*F14A*). Unless one of the bacterial species inciting enteric disease is isolated from the first plating, streak plates of bismuth sulfite and bile salts citrate agar (*F50.5*) from the broth and peptone tetrathionate medium cultures after from eighteen to twenty-four hours' incubation. The presence of some nonpathogenic microorganisms in large numbers may render the results of questionable significance and the specimen unsatisfactory.

Examine as directed for feces.

GALL BLADDER AND BILE

Remove the bile from the gall bladder with a pipette or syringe and needle, using precautions to prevent contamination. Examine the bile as outlined for duodenal drainage.

Macerate a small piece of the gall bladder wall in salt solution, taking precautions to minimize contamination. Stain a film preparation by Gram's method (p. 35). Streak plates of blood (*F43A*), Endo (*F52A*), and bismuth sulfite agar (*F51*). Transfer portions of the gall bladder suspension to 6–8 ml. of beef-extract broth (*F14A*) and to 12 ml. of peptone tetrathionate medium (*F25.5*), to which the 0.3 ml. of iodine solution has been added immediately preceding use. If pure cultures of Gram-negative bacilli are obtained in fluid media after one or two days' incubation, test for agglutination. Plate fluid cultures, if necessary, on bismuth sulfite and bile salts citrate agar (*F50.5*).

When stones are found in a gall bladder, wash one or more first in 70-percent alcohol and then in salt solution, crush in a mortar, and suspend in a small amount of salt solution. Prepare cultures as outlined for the gall bladder wall.

If a bacterial species of diagnostic significance other than common bacillary incitants of enteric disease is indicated, modify the procedure to favor its isolation and identification.

The presence of some nonpathogenic microorganisms may render the results of the examination of questionable significance and the specimen unsatisfactory.

Examine as directed for feces.

BLOOD

Make a cultural examination of blood specimens received from patients with symptoms of typhoid or paratyphoid fever.

Add the clot to 8–10 ml. of peptone bile glycerol medium (*F25*). After forty-eight hours' incubation, stain a slide preparation by Gram's method (p. 35). If Gram-negative bacilli are found, streak plates of Endo (*F52A*) and bismuth sulfite agar (*F51*). If the presence of microorganisms of possible diagnostic significance, other than the recognized bacillary incitants of enteric disease, is indicated, try to isolate and identify them. If no bacteria are found and contamination has not rendered further study impracticable, re-examine after four days' additional incubation.

If blood is received in buffered 30-per-cent glycerol solution or contains an anticoagulant, add 5–10 ml. to 8–10 ml. of peptone bile glycerol medium and examine as directed for blood clots.

Identify the microorganisms isolated as outlined for feces.

Serologic Examination

Test for Floccular and Granular Agglutination with Bact. typhosum.—(See "General Instructions," p. 264.) Dilute the serum 1:10, 1:20, 1:40, 1:80, and 1:160. To one series, add formalin-treated and to a second, alcohol-treated suspension of *Bact. typhosum* (see preparation of bacterial suspensions, p. 799). If advisable, tests may also be made with actively motile living cultures in peptone bile medium (*F23*). Shake the tubes to ensure thorough mixing and incubate at 48°–52°C. for from fifteen to twenty hours.

Record the results, and, unless agglutination occurs in a 1:80 or greater dilution, centrifuge the tests for ten minutes at 2000 r.p.m. Report the titer of floccular and granular agglutination when either occurs in a 1:40 or greater dilution before centrifuging. If agglutination occurs only after centrifuging, report this fact with a statement that this may be significant.

Floccular and granular agglutination in a 1:80 or greater dilution is rarely observed in sera other than those from typhoid-fever patients unless vaccine has been recently administered. Granular agglutination in a 1:80 or greater dilution and no floccular agglutination may occur in sera from patients with typhoid fever but this type of reaction is most commonly associated with infections with microorganisms antigenically related to *Bact. typhosum*, and thus suggests the possibility of paratyphoid fever.

Floccular agglutination in a 1:80 or greater dilution and no granular agglutination may be obtained in sera from individuals to whom vaccine has been administered or who have had typhoid fever in the past or are carriers. Agglutinative properties cannot always be demonstrated in such sera, however. Unless the blood is collected soon after onset, only a very small percentage of sera from typhoid-fever patients fails to react in dilutions of diagnostic significance with one or both of the killed suspensions.

Vi Agglutination with Bact. typhosum.—The demonstration of Vi agglutinative properties may aid in the determination of typhoid carriers (22–26).

Maintenance of Standard Vi Strains.—Maintain one or more pure Vi strains of *Bact. typhosum* on inspissated egg medium (*F15.6*) and in a frozen and dried state (see p. 90). Every three months, inoculate ten or more slants of egg medium from a previous culture by making one streak from the base to the tip of the slant. Incubate for from sixteen to eighteen hours, seal the tubes with paraffin, and store in a refrigerator. Test one of these to determine if the morphologic, cultural, and biochemical properties are characteristic of the typhoid bacillus. Also, test for agglutination in pure Vi and standard typhoid sera. Agglutination should occur in the former but not in the latter. Avoid keeping cultures or suspensions at room temperature. Place them in the refrigerator if they are not used promptly.

Technic of the Test.—When a test for Vi agglutinative properties is to be performed, inoculate one or more slants of beef-infusion agar (*F33*) from an egg medium slant and incubate for from sixteen to eighteen hours. One egg medium slant will usually suffice for the inoculation of a considerable number of agar slants. Suspend the growth from agar in salt solution to give a turbidity matching that of glass standard No. 2.5 and combine with 1:5, 1:10, 1:20, and 1:40 dilutions of the patient's serum. Incubate the tests for two hours and allow to stand overnight in a refrigerator. Record the reactions, centrifuge for ten minutes at approximately 2000 r.p.m. and record the reactions again after shaking. Compare with the suspension combined with salt solution and with specific dilutions of known serum. Prepare the latter by absorbing an agglutinating serum produced with a pure Vi strain of *Bact. typhosum* with one possessing little or no Vi antigen.

Record the reactions:

4+ complete agglutination in clear fluid
3+ definite agglutination in clear or nearly clear fluid
2+ clumps, definitely visible to the unaided eye in turbid fluid

+ questionable or trace of clumping
− no clumps; uniformly turbid suspension

Repeat the test at least once after the serum has been stored in the refrigerator for several days and report the dilution in which definite agglutination is obtained after centrifuging in at least two tests.

STANDARDIZATION AND TESTING OF MEDIA

Test each lot of freshly prepared plating medium, lactose-glucose agar, and media employed for determination of biochemical properties with standard strains of bacillary incitants of enteric disease and *Bact. coli*.

To standardize Endo agar (*F52A*) prepared with a new lot of basic fuchsin, test portions of the medium to which varying amounts (usually 0.05–0.3 ml. per 100 ml. of agar) of a saturated alcoholic solution of the dye have been added. Streak with fecal specimens containing bacillary incitants of enteric disease as well as with pure cultures of these species and *Bact. coli*. This medium is considered satisfactory only when colonies of *Bact. coli* are deep red with little or no diffusion of color through the agar and the growth of bacillary incitants of enteric disease is not inhibited.

When a new lot of brilliant green dye is to be employed in bismuth sulfite agar (*F51*), it may be necessary to test this medium prepared with varying amounts of the dye. Adjustments of other ingredients may need to be considered from time to time to compensate for variations which are at present not clearly understood. Determine the optimum amounts of dye or other ingredients by standardizing as described for Endo agar. It may be necessary to determine in a similar manner the optimum amounts of citrate and dyes to be used with different lots of agar or peptone in the citrate agar (*F44* or *F50.5*).

When a new lot of acid fuchsin is obtained for use in Andrade indicator (*F50:A*, *B*), test lactose-glucose agar containing the indicator prepared with varying amounts of the dye. Inoculate with represenitve strains of the species listed in table 13. The indicator is considered satisfactory only when the medium inoculated with members of the coliform group is red throughout and with nonlactose or slow lactose fermenting species is red in the butt only with a colorless slant after incubation for eighteen to twenty hours.

Test each lot of buffered glycerol solution (*F70*) to be used in outfits for the submission of specimens. Combine equal parts of the glycerol solution and beef-extract broth (*F14A*) and inoculate with approximately 0.2 ml. of 3- to 5-hour broth cultures of *Bact. typhosum* and of representative strains of *Salmonella* and dysentery bacilli. Store these suspensions at room temperature and streak on appropriate plating media after three days and seven days. Consider the glycerol solution satisfactory only when all the species remain viable with no appreciable decrease in numbers for seven days.

VIBRIO CHOLERAE

Outbreaks of Asiatic cholera have not occurred in the United States for many years, and therefore methods presented here for examinations for evidence of this highly infectious disease are based on the experience of others in this field, especially of those in India where much work has been done (27–32). Specimens from patients having symptoms of cholera should also be examined for bacillary incitants of enteric disease, particularly dysentery bacilli.

FECES

Microscopic Examination

Microscopic examination is of value only as a presumptive test to aid in diagnosis. Stain slide preparations of flakes of mucus from characteristic rice water stools by Gram's method (p. 35) and with aqueous alcoholic methylene blue (p. 38). If Gram-negative comma-shaped microorganisms are found, examine moist preparations. Report promptly the presence of numerous actively motile microorganisms morphologically typical of *Vibrio cholerae.*

Cultural Examination

Consider feces satisfactory for examination for *V. cholerae* only when the specimen (1) is received promptly after collection, (2) has been kept at a low temperature (6°–10°C.), or (3) is suspended in alkaline peptone water (*F29B*) or an alkaline buffer solution such as that recommended by Venkatraman and Ramakrishnan (29). Streak plates of beef-extract agar (*F49A*) adjusted to pH 8.0–8.4, alkaline coagulated-blood agar (*F38*), or alkaline egg medium (*F48*). At the same time, for purposes of control and comparison, streak plates of these media with a known culture of *V. cholerae.* Add a portion of the specimen to alkaline peptone water (*F29B*) or the bismuth sulfite medium recommended by Read (30) and, after incubation for from eighteen to twenty hours, streak plates from the surface. When microorganisms having the morphology of *V. cholerae* have not been found in slide preparations of feces, examine stained films and moist preparations of the enrichment medium after from six to eight hours.

Isolate and identify microorganisms having the morphology of *V. cholerae.* Report as *V. cholerae* a motile Gram-negative microorganism that liquefies gelatin promptly, produces indol, and reduces nitrates rapidly (cholera-red or nitroso-indole reactions, see p. 17), forms acid but no gas in medium containing glucose, mannitol, maltose, and sucrose but not in that with dulcitol, does not hemolyze goat red blood cells, and is agglutinated in a serum with specific agglutinative properties for the antigen designated as OI (28).

The significance of vibrios that hemolyze goat red blood cells but have all the other properties of *V. cholerae* designated as the "El Tor" group has not been established.

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CHAPTER 24

EXAMINATION FOR INCITANTS OF FOOD POISONING

Various species of microorganisms produce enterotoxins in certain foods under favorable conditions (1–4). Hence, the examination of such specimens should be undertaken only when pertinent clinical data are available and an epidemiologic study clearly implicates the food in question. If clinical manifestations of botulism are reported, examine at once for evidence of botulinus toxin. When symptoms of enteritis are reported, the examination of fecal specimens collected soon after the onset of the illness may be important. Certain species of *Salmonella* and of dysentery bacilli are not infrequently disseminated by means of food. It has not been determined whether some of the former give rise to enterotoxins in foods or produce toxins so promptly after ingestion that the symptoms are considered those of food poisoning. Enteritis incited by certain types of dysentery bacilli may develop within twelve to twenty-four hours after ingestion of contaminated food.

Reliable methods for demonstrating the enterotoxic properties of staphylococci, streptococci, *Proteus*, and other possible incitants of food poisoning have not been established. An estimation of the relative numbers of the various bacterial species found is very important unless the food has been kept for a considerable time under conditions favorable to the development of bacteria that may be present because of unavoidable contamination.

If the examination of food and beverages for toxic chemicals is undertaken, use the procedures outlined in Standard Methods of the Association of Official Agricultural Chemists (5).

CLOSTRIDIUM BOTULINUM (6–11)

FOOD

Observe the special precautions outlined in "General Bacteriologic Technic," for handling highly infective material (p. 4).

If food suspected of containing *Clostridium botulinum* is received in cans, open aseptically. Scrub the can with soap and hot water, allow it to stand in 2-per-cent cresol compound (*F76*) for a few minutes and dry with a sterile towel or gauze, flame, and open with a sterile can opener.

Microscopic Examination

Stain slide preparations by Gram's method (p. 35) and examine for thick Gram-positive bacilli with terminal or subterminal spores wider than the rod.

Toxicity Tests

If the specimen is solid, suspend part in approximately ten times its volume of salt solution, grinding in a mortar if necessary, and extract for one or two hours at room temperature.

Centrifuge a fluid specimen or the extract of a solid specimen. Test the supernatant for toxicity by injecting intraperitoneally 0.5 ml. of the unheated material into each of two mice and 1 ml. into each of two guinea pigs (weight not over 300 grams each), or, if slide preparations show large numbers of bacteria, test a filtrate obtained by passage through a diatomaceous earth filter. For control, heat a portion for thirty minutes in a water bath at 100°C. and inject the same number of animals with the same amounts of the heated material. Combine 2–4 ml. of the unheated supernatant fluid or filtrate with 300–400 units of botulinus antitoxin type A (contained in 1 ml. of a dilution in salt solution). Allow to stand at room temperature for one-half to one hour and inject 1 ml. of the mixture intraperitoneally into a mouse and 2–4 ml. into a guinea pig. Follow the same procedure with botulinus antitoxins type B and type E. Observe all the inoculated animals at frequent intervals for symptoms of botulism.

Unless the animals succumb within four to eighteen hours, the following symptoms are most commonly observed: extreme weakness, striking emaciation, flabby abdominal muscles, and respiratory paralysis. Increased secretion of mucus from the nose and mouth is usually induced by types A and B toxins but may not be by type E. If the specimen contains botulinus toxin, only animals that received unheated material should die within four to seventy-two hours, while those inoculated with the heated portion should remain alive. Death of the latter indicates the presence of other toxic substances. The type of botulinus toxin is determined by the protection afforded by one of the antitoxins.

At autopsy of guinea pigs with fatal botulinus toxemia the site of inoculation generally shows no significant macroscopic changes; occasionally slight infiltration or edema, with or without hemorrhages, may be observed. The abdominal organs are usually congested, with dark-colored liver and spleen; in acute intoxications, these lesions may be absent. The bladder is frequently distended. The lungs are hyperemic and may show large or small petechiae.

The possibility of types of *Cl. botulinum* other than A, B, and E must be considered when the animals are not protected by antitoxins for any of these three types.

Cultural Examination

Confirm the toxicity tests by cultural examination.

Heat a portion of the sediment of the centrifuged specimen at 60°C. (12, 13) and another at 80°C. for one-half hour to destroy vegetative forms before adding to culture media. If the material is very acid, neutralize before heating.

Streak three series of blood-agar plates (*F43A*) with the heated and unheated suspensions of the sediment of the centrifuged specimen. Incubate one series of plates under aerobic and one under anaerobic conditions at 35°–37°C. and the third set under anaerobic conditions at 25°–30°C. for forty-eight to seventy-two hours. Incubation at the lower temperature is favorable to the development of *Cl. botulinum* type E (9, 10). Include with each series a blood-agar plate inoculated with a representative strain of *Cl. botulinum* for control. Colonies of *Cl. botulinum* on blood agar are hemolytic, moist, usually flat, ameboid, and often confluent. Fish typical or suggestive colonies to semisolid agar (*F32C*) or chopped-meat medium (*F6*), incubate duplicate cultures at 25°–30°C. and at 35°–37°C. for five to ten days, and test for toxicity.

If *Cl. botulinum* is not isolated from blood-agar plates, inoculate 10- to 25-ml. amounts of semisolid medium and chopped-meat medium with 2–3 ml. of heated and unheated suspensions of the sediment from the centrifuged specimen. Incubate one series of cultures at 35°–37°C. and one at 25°–30°C. for five to ten days and test for toxicity.

FECES (14)

Dilute a large amount of the specimen with salt solution and heat a portion at 60°C. and another at 80°C. for one-half hour to destroy vegetative forms. Inoculate culture media and test, as described under specimens of food.

INCITANTS OTHER THAN CLOSTRIDIUM BOTULINUM

FOOD

Microscopic Examination

Examine films stained by Gram's method. Record the relative number of each type of microorganism present.

Cultural Examination

Streak with the specimen or a suspension of it, two series of blood-agar plates (*F43A*) and one of selective media for the isolation of bacillary incitants of enteric disease. Incubate the latter under aerobic conditions. Incubate one series of blood-agar plates under anaerobic conditions and the other in the presence of 5–10 per cent of carbon dioxide.

Estimate the relative numbers of all bacterial species present and isolate any that are of possible significance. The extent of the study of these microorganisms is largely dependent upon the results of the epidemiologic survey. Identify any significant species.

Animal Tests

Monkeys may be fed, kittens fed or inoculated intraperitoneally, or rabbits inoculated intravenously with filtrates of suspensions of the food or of cultures of the microorganisms that are thought to be of significance. A medium simulating the food in which enterotoxins were apparently produced may facilitate the demonstration of toxic substances.

FECES

Examine feces for bacillary incitants of enteric disease (p. 297) and for the species indicated to be of possible significance by the examination of food.

BLOOD

Serologic tests have not been developed as an aid in the determination of the etiologic agent in food poisoning. Separate the serum from the clot and save the former for examinations that may be desirable in view of the clinical manifestations and the bacteriologic findings.

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CHAPTER 25

EXAMINATION FOR HEMOPHILUS

HEMOPHILUS PERTUSSIS

Laboratory aids are of particular value in furnishing data that will confirm or establish the diagnosis early in the course of an outbreak or when isolated cases occur and the symptomatology is not entirely characteristic.

The examination for *Hemophilus pertussis* should be undertaken immediately after collection of the specimen. Therefore, material submitted on swabs and sputum sent from a distance are considered unsatisfactory if *H. pertussis* is not isolated.

COUGH SPRAY, SPUTUM, AND NASOPHARYNGEAL MUCUS

Cultural Examination

For isolation and subculture, use freshly prepared potato-infusion blood agar (*F56A*) or that that has been stored in a refrigerator for not longer than two weeks, preferably one week or less. If freshly collected sputum is received, wash it by suspending in approximately 10 ml. of salt solution in a Petri plate and drawing it in and out of a 2-ml. syringe (fig. 34, p. 272) before streaking the plates.

Nasopharyngeal swabs may be especially useful in collecting material from infants and other noncooperative patients (1–3). For this purpose, a small cotton swab on a thin flexible wire is satisfactory. The swab on which the specimen has been collected should be placed in a sterile empty tube or in one containing a short slant of potato-infusion blood agar (1) and transported to the laboratory as promptly as possible. Immediately on receipt, the specimen should be streaked on a potato-infusion blood-agar plate as directed for grossly contaminated material, p. 13. Incubate all plates immediately and examine after two, three, four, and six days. When small, raised, glistening, pearly, hemolytic colonies are found, fish them to slants or plates of the medium used for isolation. Determine the morphology of the microorganisms isolated. If small oval Gram-negative bacilli are present, inoculate potato-infusion blood agar (*F56A*), beef-infusion-agar (*F33*), and coagulated-blood-agar medium (*F37* or *F55.5A*).

The failure of hemophilic bacilli to grow on beef-infusion agar is an aid in their identification. *Hemophilus pertussis* when freshly isolated grows very poorly on coagulated-blood-agar medium whereas *Hemophilus influenzae* grows well.

Perform a slide-agglutination test (4). Suspend growth from a 20- to 48-hour culture in a drop of salt solution on a slide. Mix several loopfuls of the suspension with an equal amount of (1) a specific dilution of known serum for *H. pertussis* and (2) salt solution. The results are significant only when definite clumping occurs in the former and not in the latter. Confirm doubtful reactions by a tube-agglutination test, see p. 758.

HEMOPHILUS INFLUENZAE

While the incitant of influenza has been shown to be a virus, *Hemophilus influenzae* may induce serious types of infection, such as meningitis.

Cultural Examination

When an examination for *Hemophilus influenzae* is indicated, plate the specimen on coagulated-blood agar (*F37* or *F55.5A*) or on blood agar (*F43A*). Colonies of this microorganism are smooth, flat, grayish, translucent, and, when well-isolated on blood agar, much smaller than when near those of other bacteria. When stained preparations from such colonies show pleomorphic Gram-negative bacilli, fish them to coagulated-blood agar (*F37* or *F55.5A*) and beef-infusion agar (*F33*). *Hemophilus influenzae* will grow on the former but not on the latter.

HEMOPHILUS DUCREYI

The incitant of chancroid is the *Hemophilus ducreyi*. It is often difficult to find in films prepared from the lesion. Cultural tests require special medium and the microorganism cannot always be isolated. Examinations for evidence of syphilis are also important since the primary lesions of both infections may resemble each other or the patient may have acquired both infections.

Microscopic Examination

Stain film preparations from the lesions by the Gram-method (p. 35) or preferably with Barritt's modification of Pappenheim's stain (p. 38). If long chains of thick Gram-negative bacilli or those stained red by the Pappenheim method are found, they may be reported as characteristic of the Ducrey bacillus. Not infrequently, however, these microorganisms are not seen and other Gram-negative bacilli present in the lesion due to contamination may be confusing.

Make also direct examinations of chancre fluid and pus from a bubo for *Treponema pallidum*, see p. 335.

Cultural Examination (5–7)

Since material from an open lesion is usually so contaminated as to render cultural procedures unsatisfactory, whenever possible secure pus from an unruptured bubo by aspiration under aseptic precautions. Inoculate slants of beef-infusion agar (*F33*) or tubes of semisolid agar (*F36*, base) to the surface of each of which has been added 1 ml. of fresh defibrinated rabbit's blood. Incubate all cultures for forty-eight hours. If growth is not obtained, reincubate and reexamine after one week. Colonies of *H. ducreyi* develop in the blood and at the point of contact of blood and agar. In stained preparations, the microorganisms appear as short, plump, Gram-negative rods occurring in pairs, groups, and parallel chains.

If subcultures are made on rabbit-blood agar (*F43A*), the colonies are small, gray, convex, and dull with entire edges. The whole colony can be pushed easily over the surface of the medium. Use beef-infusion agar or semisolid agar with fresh rabbit's blood for preserving cultures. Transfer at weekly intervals and store in the icebox after incubation.

Serologic Examination

Examine blood for evidence of syphilis as directed for serologic tests for syphilis, p. 361.

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CHAPTER 26

EXAMINATION FOR BRUCELLA AND OTHER MICROORGANISMS COMMONLY ASSOCIATED WITH ANIMAL HOSTS

Observe the special precautions outlined in "General Bacteriologic Technic" for handling highly infective material, p. 4.

BRUCELLA

Brucella abortus is the incitant of almost all of the undulant fever that occurs in New York State (1). It can seldom be isolated from the blood stream unless the specimen is obtained during an exacerbation. Furthermore, since these microorganisms may remain viable in whole blood for only a short time after collection, cultural examination and animal tests should be undertaken promptly. They have also been isolated from feces, urine, bile, uterine discharge, and pustules, and from foci that may persist after apparent recovery from undulant fever. The examination of such specimens should be undertaken as soon as possible after they are procured. *Brucella abortus* is often agglutinated in sera from patients having tularemia but in an appreciably lower dilution than *Bacterium tularense*. The latter is seldom agglutinated in sera from patients having undulant fever.

BLOOD

Cultural Examination

Inoculate semisolid agar (*F32C*) and blood-agar plates (*F43A*) as directed under the cultural examination of blood (see "General Instructions," p. 263). Incubate cultures on solid media in the presence of 5–10 per cent of carbon dioxide. If growth is not observed at the end of a week, transfer 0.5-ml. and 3-ml. amounts of each semisolid-agar culture to a blood-agar slant and a bottle containing 50 ml. of semisolid-agar medium, respectively. Re-examine at weekly intervals for at least six weeks unless a member of the genus *Brucella* or some other significant species is isolated or the presence of contaminating microorganisms renders further examination impracticable.

When small, nonmotile, Gram-negative bacilli morphologically typical of *Brucella* are isolated, test for agglutination in specific dilutions of known serum.

Serologic Examination

Macroscopic Tube-Agglutination Test. (See "General Instructions," p. 264.) Prepare 0.6 ml. of a 1:5 dilution of the serum, inactivate by heating at 55°–56°C. for thirty minutes, and make 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 dilutions. Combine with alcohol-treated suspension of *Br. abortus* (see preparation of bacterial suspensions, p. 802). Incubate the tests at 48°–52°C. for four hours, allow to stand overnight in the refrigerator, and record the reactions. Centrifuge for ten minutes at approximately 2000 r.p.m. and record the reactions again (see p. 265). If definite agglutination occurs in a 1:20 or greater dilution only after centrifuging, repeat the test, report the findings, and request another specimen. Otherwise, report the greatest dilution in which definite agglutination occurs before centrifuging.

Animal Tests

Cultural examination is preferable to animal tests for the demonstration of *Brucella* in blood. The following procedure may be employed, however, if the inoculation of animals seems advisable.

Inject a 250- to 300-gram guinea pig intraperitoneally with 2–5 ml. of blood to which an anticoagulant has been added, or of clotted blood comminuted and suspended in salt solution. If the animal is alive at the end of eight weeks, remove 1–2 ml. of blood from the heart. Test the serum for agglutination with an alcohol-treated suspension of *Br. abortus.*

If a reaction occurs, chloroform and autopsy the guinea pig. If no agglutination is obtained, keep the animal under observation and bleed at intervals of two to four weeks. If no agglutination occurs in blood procured four or five months after inoculation, kill and autopsy the animal.

Make stained preparations and cultures on blood-agar slants from any apparent foci and from the heart's blood, spleen, liver, bile, and urine. Remove the bile and urine with syringes and hypodermic needles. The gross abnormalities in infected guinea pigs may be limited to a slight enlargement of the spleen. This organ is often studded with caseous nodules and lymph glands are frequently enlarged. Other foci may develop including inflammatory lesions in the regions of the joints. (See fig. 38.)

URINE, FECES, AND OTHER SPECIMENS

When the examination of such material is undertaken, each must be considered individually in the light of the clinical data furnished. If the specimens are not contaminated, examine as directed for blood. Otherwise, select tests appropriate to the type and condition of the material.

BACTERIUM MALLEI

Bacterium mallei is the incitant of glanders, which is primarily a disease of horses, but which can be transmitted to man. The microorganism is found in the nasal secretions, pus from nodules, blood, and at times in the urine, saliva, and milk of infected animals. In man, the mode of infection is usually through an abrasion of the skin, but may be through the mucosa of the mouth and nose. A nodule appears at the site of infection, accompanied by lymphangitis and swelling. A general pustular eruption may occur. While the disease is usually acquired from contact with horses, it may be transmitted from man to man.

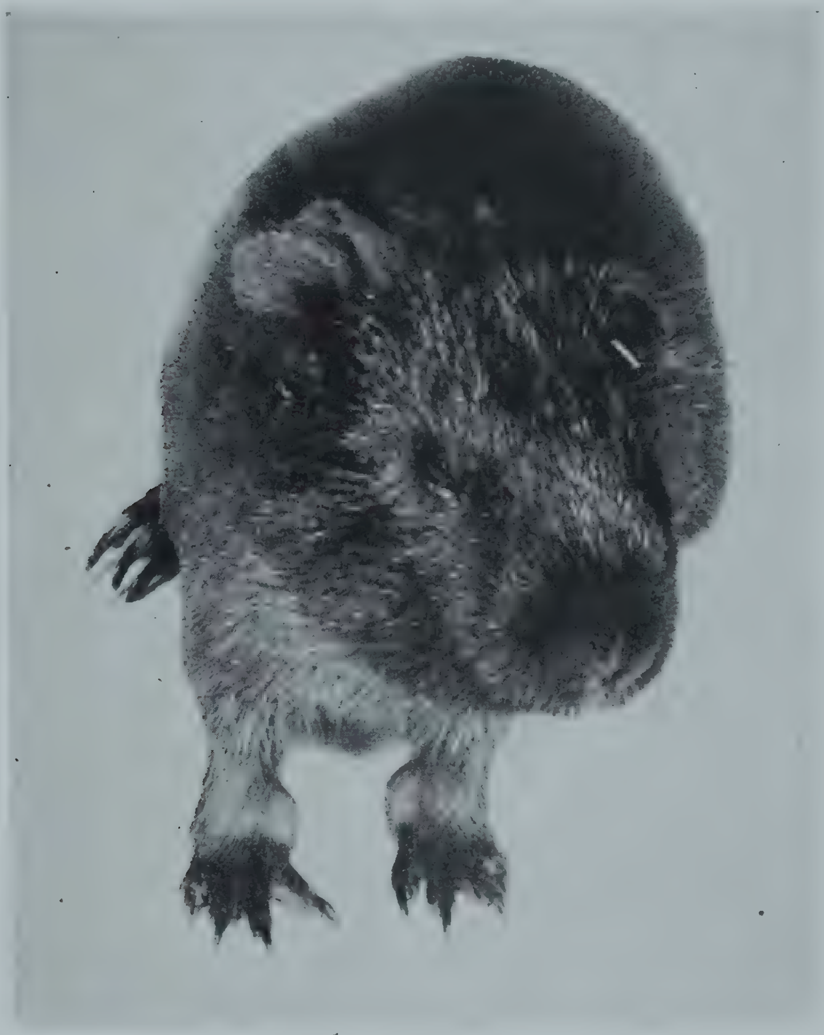

Fig. 38
Guinea Pig with Br. Abortus Infection: Foci in Joints

DISCHARGE

Microscopic Examination

Stain films by Gram's method (p. 35) and with aqueous alcoholic methylene blue (p. 38). If material is not available for cultural tests, report the results with an explanation of the limitations of the microscopic examination.

Cultural Examination

Streak blood-agar (*F43A*) plates. After twenty-four to forty-eight hours' incubation, colonies of *Bact. mallei* are usually round, translucent, grayish-yellow and 0.5–1 mm. in diameter. When a pure culture is obtained, study its morphologic, cultural, and biochemical properties.

Bacterium whitmori, *Actinobacillus lignieresi*, *Bacterium enterocoliticum* (2, 3) and species of the genus *Pasteurella* have many properties in common with *Bact. mallei*. When morphologic, cultural, and biochemical properties suggest any of these microorganisms, make a systematic study of the strain to determine its identity, if possible.

Bacterium mallei is a small, nonmotile, granular, Gram-negative, straight, or slightly curved rod. It grows well but slowly on most of the ordinary culture media. Slight acid reactions may be produced in media containing salicin and glucose and in milk. Gelatin may be liquefied slowly. The appearance of the growth on potato (*F27*) is characteristic and is an important factor in its identification. After incubation for forty-eight hours, a yellowish, thick viscous film appears which, during the next few days, becomes brown. The potato in the region of the growth turns dark.

Animal Tests

Inject the original specimen or a mixed culture subcutaneously into a full-grown male guinea pig; inject pure cultures intraperitoneally.

Enlargement of the testicles usually takes place within two or three days after intraperitoneal, and within one to two weeks after subcutaneous inoculation. Death may occur in one to eight weeks but generally during the third or fourth week. Grayish yellow nodules are usually present in the lungs, spleen, liver, and testicles. Microorganisms other than *Bact. mallei*, particularly *Bact. whitmori*, *Actinobacillus lignieresi*, and *Bact. enterocoliticum* may produce similar lesions in guinea pigs; therefore histologic examination of the organs, as well as a study of the cultural and biochemical properties of the microorganisms, is important.

If a guinea pig inoculated subcutaneously dies without characteristic lesions, inject another male guinea pig with a portion of lymph gland from the first.

PASTEURELLA PESTIS (4, 5)

Plague is principally a disease of rodents, the incitant of which, *Pasteurella pestis*, is transmitted, except in the pneumonic form, by fleas and probably other blood-sucking insects. The microorganism is harbored chiefly by the rat, but ground squirrels and other rodents have also been shown to be the source of the infection. Plague has been proved to be endemic in wild rodents in some districts in California, and infected animals are occasionally reported in other western states. Thus, the possibility of the occurrence of the disease in New York State must be considered. While suggestive findings may be obtained by morphologic examination of exudate from a bubo, the results of bacteriologic and animal tests are necessary to identify *Past. pestis.*

SPUTUM AND DISCHARGE FROM BUBOES

Microscopic Examination

Stain slide preparations by Gram's method (p. 35) and with aqueous alcoholic methylene blue (p. 38). Gram-negative bacilli that have a tendency to stain more intensely at the poles suggest *Past. pestis.* The microorganism, however, cannot be identified by means of microscopic examination alone.

Cultural Examination

Streak plates of blood agar (*F43A*) and of 3-per-cent sodium-chloride agar (*F34B′*). Involution forms of *Past. pestis* develop on the latter. Inoculate broth and beef-extract gelatin (*F31*). *Past. pestis* produces a delicate pellicle with pendant thread-like growth (stalactites) in the former and does not liquefy the latter. Test for agglutination with known serum.

Animal Tests

Inoculate one guinea pig subcutaneously and rub some of the material on a dry shaved area on the abdomen of another.

If the inoculum contains *Past. pestis*, the animals usually die within three to five days. At autopsy, enlarged regional glands, small necrotic foci in the liver, and miliary nodules in the spleen are found. The bacilli are present in large numbers in the enlarged lymphatic glands, liver, spleen, and blood. Similar lesions may be induced by other species, particularly *Past. pseudotuberculosis* and *Bact. enterocoliticum.*

BLOOD

Cultural Examination

Streak plates of blood agar (*F43A*) and inoculate beef-infusion broth (*F2A*) or semisolid agar (*F32C*). Streak the latter on blood agar after forty-eight hours' incubation and reincubate in case further examination seems desirable. Identify the microorganisms isolated as outlined for sputum and discharge.

Animal Tests

Guinea pigs may be inoculated either with the specimen or cultures isolated from it, as with sputum or discharge.

Serologic Examination

Serum may be tested for agglutination with *Past. pestis*. Use a suspension of this microorganism in salt solution containing 0.5-per-cent formalin and having a turbidity comparable to glass standard No. 2.5. If necessary to remove clumps from the agglutinating suspension, filter through nonabsorbent cotton.

BACTERIUM TULARENSE (6, 7)

Bacterium tularense is transmitted from animal to animal and from animal to man by blood-sucking insects as well as by direct contact in handling and dissection of animals. Since tularemia is relatively rare in New York State, laboratory examinations are particularly important as an aid in diagnosis. Agglutinative properties for *Bact. tularense* may not be demonstrable in patient's serum until the third or fourth week of illness and they may persist for years, probably for life. Sera from patients having tularemia often agglutinate *Brucella abortus* but in an appreciably lower dilution than *Bact. tularense*. The latter is seldom agglutinated in sera from patients having undulant fever.

PUS OR DISCHARGE FROM GLANDS

Microscopic Examination

Stain slide preparations by Gram's method (p. 35). Record the number and character of the microorganisms present. *Bact. tularense* is stained with difficulty in body fluids or tissue. The microorganism, however, cannot be identified by means of microscopic examination alone.

Cultural Examination

If small Gram-negative bacilli are found on microscopic examination, streak cystine blood-agar plates (*F35C*) (8) and incubate for three weeks unless growth appears sooner. However, this species does not usually develop on artificial culture media inoculated directly with specimens. Determine the presence of other microorganisms by appropriate bacteriologic examinations. Test small Gram-negative bacilli that grow only on medium containing cystine and blood for agglutination in *Bact. tularense* serum. Incubate the tests for two hours and leave in the refrigerator overnight before reading the results.

Animal Tests

Inject one guinea pig subcutaneously with 0.5–3 ml. of the specimen and rub some of the material on the skin of the abdomen of another which has been abraded by shaving with a dull razor. As a rule, guinea pigs inoculated with *Bact. tularense* die in five to ten days. The lymph glands are usually enlarged and filled with caseous material and the liver and spleen studded with numerous small, white, caseous nodules.

If no characteristic lesions are observed, place a portion of the spleen in 50-per-cent glycerol for further bacteriologic study. Also examine sections of these organs and, if lesions suggestive of tularemia are found, inject guinea pigs subcutaneously. Continue to reinfect guinea pigs until the incitant is identified. If the animals are alive two weeks after inoculation, bleed from the heart, and autopsy. Cultures of *Bact. tularense* on cystine-blood agar may be obtained from lesions of guinea pigs and should be incubated for at least three weeks, unless growth appears sooner.

BLOOD

Serologic Examination

Macroscopic Tube-Agglutination Test.—(See "General Instructions," p. 264 and "General Bacteriologic Technic," p. 19. Dilute the serum 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160 and combine with a formalin-treated suspension of *Bact. tularense* (see preparation of bacterial suspensions, p. 803). Incubate the tests for two hours, leave in the refrigerator overnight and record the results. Centrifuge for ten minutes at approximately 2000 r.p.m. and record the reactions again (see p. 265). If definite agglutination occurs in a 1:20 or greater dilution only after centrifuging, repeat the test, report the findings, and request another specimen. Otherwise, report the greatest dilution in which definite agglutination occurs before centrifuging.

BACTERIUM ENTEROCOLITICUM (2, 3)

Bacterium enterocoliticum has been isolated from localized infections and from ulcerative lesions in the intestines and from feces from patients having enterocolitis.

Although the microorganism has not yet been isolated from natural infections in animals, the fact that mice are readily infected by feeding suggests the possibility that rodents may serve as a reservoir for the microorganism.

FECES, PUS, OR OTHER DISCHARGE

Cultural Examination

Streak plates of blood (*F43A*), Endo (*F52A*), and bile salts citrate agar (*F44* or *F50.5*). After eighteen hours' incubation, smooth colonies of *Bact. enterocoliticum* are opaque and vary from the size of a pinpoint to about 0.5 mm. in diameter. Rough colonies with crenated edges develop very readily and are larger and more translucent than smooth colonies.

This microorganism is a small Gram-negative rod with a tendency to bipolar staining, and is motile when grown at 18°–30°C. It can be identified on the basis of its biochemical reactions and agglutination in known serum (see table 13, p. 300). The smooth phase can be maintained by passage through mice for which it is particularly pathogenic. Transition to the rough phase is accompanied by loss of virulence.

Animal Tests

Inject mice subcutaneously or intraperitoneally with a portion of the specimen or a culture. When large numbers of contaminating bacteria are present as in feces, also feed mice. Mice infected with *Bact. enterocoliticum* usually die within two to ten days but may survive for as long as a month. Most strikingly characteristic are the numerous miliary necrotic nodules varying in size from a pinpoint to areas 3–5 mm. in diameter scattered throughout the peritoneal cavity, liver and sometimes the other viscera, and the diaphragm. The histologic changes closely resemble those in mycotic infections.

BLOOD

Perform cultural and serologic examinations (see "General Instructions," p. 263). Blood from patients infected with this species has seldom been available for examination and hence the fact that the microorganism has not been isolated from this type of specimen is not significant.

ERYSIPELOTHRIX RHUSIOPATHIAE

Swine erysipelas occurs as an occupational disease in man, among those who handle hogs or decaying animal material such as bone, fish, etc. (9). The skin lesion develops usually on the fingers and spreads towards the wrist. It may assume a vesicular or hemorrhagic appearance with enlargement of the adjacent lymph nodes.

DISCHARGE

Microscopic Examination

Stain slide preparations by Gram's method (p. 35). *Erysipelothrix rhusiopathiae* is a small, slender, Gram-positive bacillus. In the attenuated form, it may appear in long chains and interlaced filaments. The microorganism, however, cannot be identified by means of microscopic examination alone.

Cultural Examination

Streak the material on blood agar (*F43A*). Colonies of the virulent form are minute, round, convex, and water-clear with a glistening surface;

colonies of the attenuated form are larger and less convex, with a granular appearance and irregular edges.

Animal Tests

Inject a mouse intraperitoneally or subcutaneously with some of the specimen. If *E. rhusiopathiae* is present, the animal usually dies in two or three days. The subcutaneous tissues are congested, the spleen is enlarged, and the lungs are bright red and edematous. The microorganism can be isolated from the organs.

SKIN

If excised portions of skin are received, grind in a mortar with salt solution and examine as described for discharge.

BACILLUS ANTHRACIS

Infections with the anthrax bacillus are usually incurred by handling animal products such as hides, hair, or wool especially those that are imported. Laboratory examination is important since prompt treatment is essential.

DISCHARGE

Microscopic Examination

Stain films by Gram's method (p. 35). The presence of large Gram-positive bacilli furnishes presumptive evidence of *Bacillus anthracis*. The microorganism can be identified, however, only by cultural and animal tests.

Cultural Examination

Inoculate beef-infusion broth (*F2A*) and beef-infusion-agar (*F33*) slants and plates. After incubation for eighteen to twenty hours, examine the cultures for large Gram-positive nonmotile bacilli that produce characteristic "Medusa head" colonies on agar, and "cotton fluff" sediment in broth.

Animal Tests

Inject guinea pigs and mice subcutaneously on the back or hind leg (10) with a portion of the specimen or of a culture from it suspended in salt solution. Animals inoculated with *B. anthracis* usually die in one to four days and this microorganism can be demonstrated in cultures and stained preparations from the spleen, liver, and blood. If animals inoculated with material containing microorganisms having the characters of *B. anthracis* do not die, inoculate others.

HAIR, BRISTLES, ETC.

Prepare an emulsion of part of the material received by cutting it in fine pieces and grinding in a mortar with a small amount of salt solution. When a shaving brush is under investigation, examine a piece of the stub in addition to the hair. Heat a portion of the emulsion at 79°–81°C. for fifteen minutes and examine microscopically, culturally, and by animal inoculation both the heated and unheated portions as described for discharge.

STREPTOBACILLUS MONILIFORMIS (11, 12)

Infection with *Streptobacillus moniliformis* is usually transmitted to man by the bite of a rat although other animals are occasionally involved. In rare instances epidemics incited by this microorganism, unassociated with rat bite, have been reported (13). The clinical manifestations of infection with *Spirillum minus* (p. 342) frequently resemble so closely those induced by *S. moniliformis* that differentiation can be accomplished only by isolating the inciting microorganism.

BLOOD AND JOINT FLUID

Only freshly collected blood and joint fluid are satisfactory for examination, since *S. moniliformis* remains viable only for a very short time in such materials. For culturing blood, inoculate semisolid meat-infusion agar (*F32C*). For culturing joint fluids and for subcultures, use beef-infusion broth (*F5A*) to which has been added 20 per cent of serum (sheep, rabbit, horse, cow, or human), 20 per cent of ascitic fluid, or 5 to 10 per cent of blood (sheep, rabbit, or human) (13). Inoculate white mice intraperitoneally with freshly drawn blood since these animals are susceptible to infection with *S. moniliformis* as well as *Spirillum minus*, the incitant of another form of rat-bite fever (see p. 342).

Streptobacillus moniliformis is an extremely pleomorphic Gram-negative nonmotile bacillus that grows in floccular masses. Blood becomes darkened near the colonies. Cultures on solid media develop best in the presence of 5–10 per cent carbon dioxide. Viability and virulence are lost rapidly on artificial culture media; consequently cultures should be transferred every three or four days. The virulence may be maintained by mouse passage.

In stained films of cultures grown on solid medium, occasional filaments with spherical, oval, fusiform or club-shaped swellings are seen. Bacillary forms, filaments, and globular bodies are found in moist preparations of 3- or 4-day cultures in serum broth. These globular bodies have thin flexible walls that give the colony the appearance of a mass of foam. Examine the moist preparations either over dark-field illumination or with the high-power dry objective. In the latter case, the addition of a loopful of 1-percent aqueous methylene blue may aid in the study of details.

Mice inoculated intraperitoneally with virulent cultures of *S. moniliformis* usually die within forty-eight hours and the microorganism can be isolated from the heart's blood or peritoneal fluid. Subcutaneous inoculation gives rise to a chronic infection characterized by enlargement of the joints.

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CHAPTER 27

EXAMINATION FOR INCITANTS OF GAS GANGRENE AND TETANUS

CLOSTRIDIUM WELCHII

The results of laboratory tests will seldom be available soon enough to serve as an aid in determining treatment for gas gangrene. Consequently, the physician must depend upon clinical and X-ray findings as guides in the treatment of the case.

When examining specimens for *Clostridium welchii*, observe the special precautions outlined in "General Bacteriologic Technic" for handling sporeforming microorganisms, p. 4.

DISCHARGE

Slide preparations as well as some of the material on a swab should be available for the examination for *Cl. welchii.*

Microscopic Examination

Stain the slide preparations by Gram's method (p. 35). Large Gram-positive bacilli suggest the presence of *Cl. welchii.* Few spores will usually be found if the films have been prepared promptly after collection of the exudate.

Cultural Examination

Heat a part of the exudate suspended in salt solution for fifteen minutes in a water bath at 79°–81°C. Inoculate in duplicate, with heated and unheated portions, blood-agar plates (*F43A*), chopped-meat medium (*F6*), and, if the microscopic examination has indicated the presence of *Cl. welchii*, deep tubes of glucose-beef-infusion agar (*F35A*), and milk (*F18A*) in fermentation tubes. Incubate one set of blood plates in the presence of 5–10 per cent carbon dioxide to detect the presence of contaminating aerobic bacteria and the other under anaerobic conditions.

Clostridium welchii produces a large amount of gas in glucose-beef-infusion agar and "stormy" fermentation of milk after twelve to twenty-four hours' incubation. The colonies on blood agar are smooth, round, raised, and produce hemolysis. For the identification of other clostridia associated with gas gangrene, the procedures outlined by Hall (1), Robinson and Stovall (2), and Reed and Orr (3) may be helpful.

CLOSTRIDIUM TETANI

The examination of specimens for *Clostridium tetani* is of little value as an aid in diagnosis since this is dependent on the demonstration of toxin production and requires a week or ten days, whereas characteristic clinical symptoms develop early.

When examining specimens for *Cl. tetani*, observe the special precautions outlined in "General Bacteriologic Technic" for handling sporeforming microorganisms, p. 4.

Microscopic Examination

Stain slide preparations by Gram's method (p. 35) and examine for Gram-positive bacilli with spherical terminal spores.

Cultural Examination

Inoculate blood-agar plates (*F43A*) and chopped-meat medium (*F6*). Incubate one set of blood plates in the presence of 5–10 per cent carbon dioxide to detect the presence of contaminating aerobic bacteria and the other under anaerobic conditions. Examine the plates after forty-eight hours for fine spreading colonies with delicate fern-like projections. If Gram-positive bacilli with spherical terminal spores are found in the chopped-meat medium after forty-eight hours, re-incubate it for five to seven days and test for the presence of toxin. Also, heat a portion of the chopped-meat culture at 79°–81°C. for thirty minutes and inoculate other tubes of this medium. Inoculate chopped-meat medium from colonies having the characteristics of *Cl. tetani* on the blood-agar plates.

Animal Tests

Inject guinea pigs intramuscularly in the thigh with 2 ml. of the filtrate or supernatant fluid from a chopped-meat culture. Observe the animals for symptoms of tetanus. (See "Production and Standardization of Tetanus Toxin," p. 634.) If, when an animal dies, confirmatory tests are considered desirable, prepare cultures from the site of inoculation and examine for *Cl. tetani*.

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CHAPTER 28

EXAMINATION FOR TREPONEMA PALLIDUM AND OTHER SPIROCHETES

Spirochetes are the incitants of some of the most widespread diseases, which, from a practical standpoint, may be classified into five groups: Syphilis and allied diseases such as yaws, pinta, and bejel, incited by *Treponema pallidum* or microorganisms having almost identical morphology; acute infectious jaundice (Weil's disease), incited by *Leptospira icterohaemorrhagiae*; rat-bite fever, by *Spirillum minus*; relapsing fever, by *Treponema recurrentis*; fusospirochetosis (Vincent's angina, bronchopulmonary spirochetosis, etc.), by spirochetes and fusiform bacilli.

A clinical diagnosis of yaws (1), bejel (2), and pinta (3) should, if possible, be confirmed by the demonstration of treponema in exudate from the surface lesion by dark-field examination. Treponema may also frequently be found in sections of the epidermis (see "Histologic Procedures," p. 555). Serologic tests for evidence of syphilis should likewise be performed (4).

TREPONEMA PALLIDUM

A blood specimen should be collected to be examined for evidence of syphilis at the time that material is submitted for dark-field examination.

FRESHLY COLLECTED EXUDATE

Demonstration of *Treponema pallidum* is essential to the early diagnosis of syphilis and depends upon the proper collection of the material. Consider the specimen unsatisfactory when an antiseptic has been used or when the material is bloody or purulent. When collecting a specimen, wear rubber gloves and take the usual precautions for handling highly infective material. To remove contaminating microorganisms, wash the suspected lesion with salt solution, using gauze or cotton to remove the surface exudate. Lightly curette the surface of the suspected chancre or lesion, remove the blood with gauze, and then squeeze the surrounding tissues until a drop of serum containing few or no red blood cells collects.

With a capillary pipette, transfer a drop of this serum to a clean glass slide, place a cover glass over it, and ring the edge of the cover glass with vaseline to prevent evaporation. Examine by dark-field illumination, see fig. 39.

If *Trep. pallidum* is not found after several preparations have been ex-

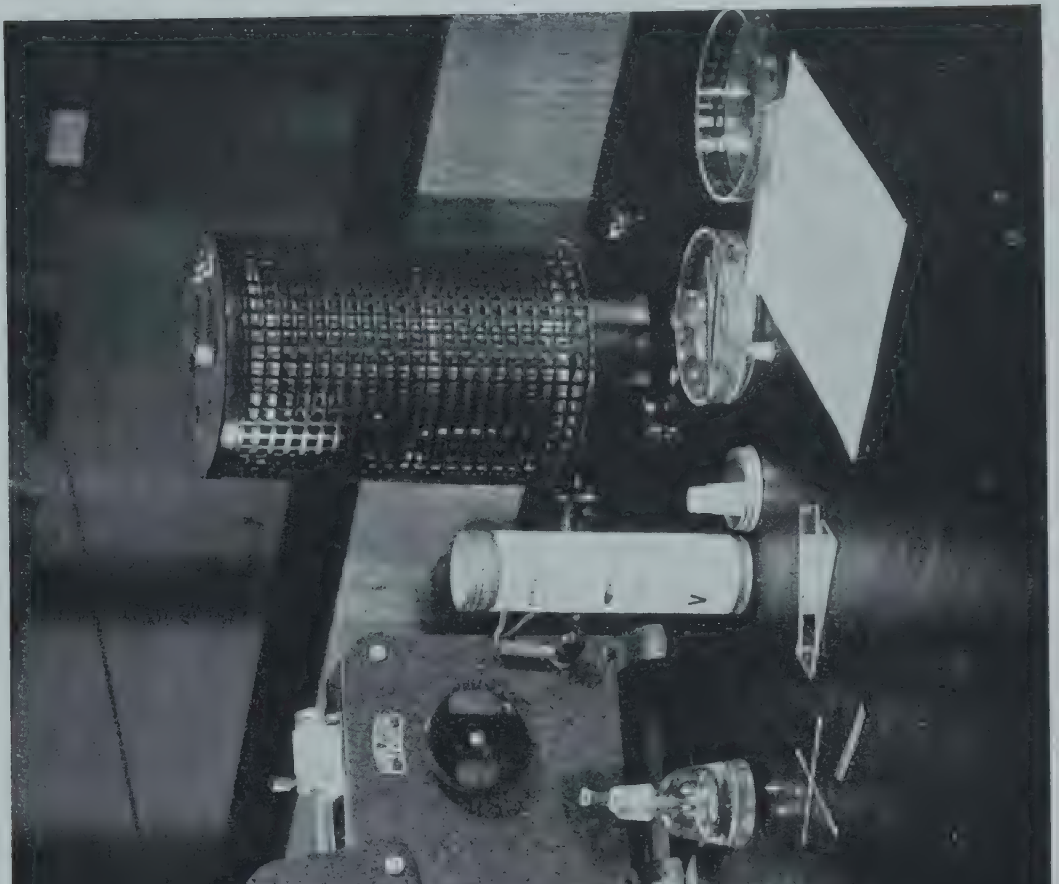

Fig. 39

Dark-Field Equipment for Examination for Treponema Pallidum amined and if glands near the lesion are palpable, examine fluid aspirated from them. To do this, draw about 0.5 ml. of salt solution into a small syringe to which is attached a 20- to 22-gauge needle 1 inch long. After the skin has been sterilized, hold the gland firmly and pierce with the needle. Force the salt solution into it, moving the point of the needle about slightly while doing this, then aspirate as much fluid as possible and examine by dark-field illumination.

Certain of the spirochetes found in nonsyphilitic lesions of the mouth resemble *Trep. pallidum* so closely that the results of microscopic examinations of exudate from the mouth or throat are of questionable diagnostic significance. In such circumstances, and if *Trep. pallidum* cannot be found in fluid from an enlarged regional lymph node, a series of specimens of blood should be submitted for serologic tests for evidence of syphilis.

EXUDATE SUBMITTED THROUGH THE MAIL

When for any reason an immediate dark-field examination of a suspected chancre cannot be undertaken, fluid collected from the lesion may be mailed to the laboratory. A satisfactory outfit for this purpose consists of sterile capillary tubes similar to those used for smallpox vaccine. They are encased in a glass tube of suitable size. A small amount of beeswax-vaseline mixture (*F80*) is provided for sealing the tubes. (See Diagnostic Outfits, "Appendix C," p. 917). Fluid from the lesion is collected in the capillary tubes; each end is then thrust into the wax to seal the opening (fig. 40); and the specimens are placed in the large glass tube before the outfit is mailed to the laboratory.

Upon receipt, protect the capillary with brown paper or gauze, then either break each end of the tube with a hemostat just beyond the wax seal and force the fluid from it with a small rubber bulb of the type that accompanies packages of smallpox vaccine, or break one end of the tube just beyond the wax seal and press the other end into the small bottle of wax until a sufficient amount of fluid is obtained for examination.

Although *Trep. pallidum* loses its active motility shortly after the specimen is collected, certain of its jerky movements are retained for a considerable time. The microorganism can be readily recognized in specimens in capillary tubes for two or three days after collection, and sometimes even several weeks later. If the lesion has been treated with an antiseptic or for any other reason *Trep. pallidum* is not found, the aspiration of fluid from enlarged regional glands should always be undertaken.

When sufficient material is available, prepare thin films of exudate and stain by Fontana's (p. 34) or Giemsa's (p. 34) method for the museum and other demonstration. *Treponema pallidum* is colored brown to black with Fontana's stain and rose red with Giemsa's.

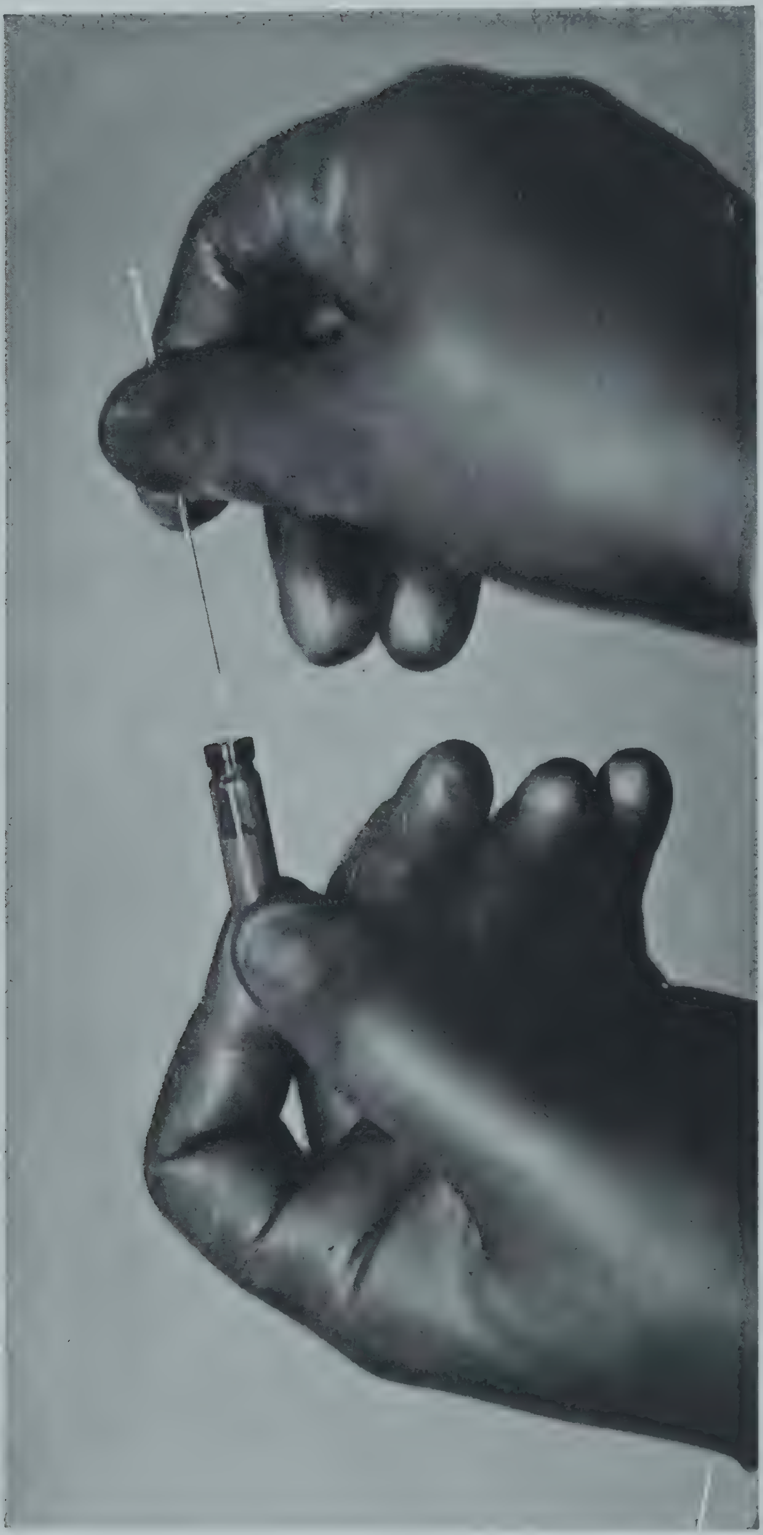

Fig. 40

Sealing Capillary Tube with Wax

TISSUE

Examine tissue obtained at biopsy or in post-mortem examination as described in "Histologic Procedures," p. 539.

LEPTOSPIRA ICTEROHAEMORRHAGIAE (5, 6)

Leptospira icterohaemorrhagiae may be found in the blood during the first three or four days of the illness, in the urine after the fifth day, and in the cerebrospinal fluid when the meninges are involved. Agglutinative properties for this species can usually be demonstrated in the blood after the ninth day of illness and may persist for years after recovery.

Leptospira canicola (7, 8), reported to be the incitant of jaundice in dogs and, occasionally, in man, is said to differ from *Lepto. icterohaemorrhagiae* in serologic reactions and pathogenicity for guinea pigs.

URINE

A satisfactory specimen of urine is very important. If it is strongly acid or alkaline, leptospirae are destroyed. If practicable, an appropriate drug should be administered to make the urine neutral when voided. About 50 ml. should be collected with aseptic precautions and delivered to the laboratory within an hour.

Since urine frequently contains nonpathogenic spirochetes of various types, a search for *Leptospira icterohaemorrhagiae* either by dark-field examination or in stained films from the sediment is not recommended as the procedure of choice. Animal test is the most reliable method.

Centrifuge 20–30 ml. of urine at 3000 r.p.m. for one-half hour and decant the supernatant.

Microscopic Examination

Make slide preparations of the sediment and stain by Gram's method (p. 35). Examine also moist preparations by dark-field illumination for leptospirae.

Chemical Examination

Test the supernatant fluid for sugar, albumin, and bile. (See "Examination of Urine," p. 514.)

Cultural Examination

If the specimen has been collected with a catheter, inoculate three tubes of rabbit serum semisolid agar (*F34E*), placing a loopful of the sediment in each. Incubate at approximately 28°C. and examine by dark-field illumination at weekly intervals for one month, unless actively motile leptospirae are demonstrated earlier. As soon as these microorganisms are found in moderately large numbers, transfer about 0.1 ml. of the top layer of the culture to two or three tubes of fresh medium.

Animal Tests

Suspend the remainder of the sediment in 5 ml. of broth and inoculate two young light-colored guinea pigs weighing 100–150 grams, intraperitoneally with 1–2 ml. each. If many contaminating microorganisms are present, inoculate the animals subcutaneously.

Examine the animals daily for jaundice in the skin and mucous membranes, particularly the sclerae. Take the temperature daily and when a marked rise is noted, anesthetize the animal and withdraw 2 or 3 ml. of blood from the heart. Add a few crystals of sodium citrate or equal parts of 2-per-cent sodium citrate in salt solution, shake to prevent clotting, and examine by dark-field illumination. Chloroform and autopsy guinea pigs (a) if jaundiced one week or ten days after inoculation; (b) when leptospirae are found in the heart's blood; and (c) if no evidence of infection with this species has been demonstrated two weeks after inoculation. Emulsions of tissues from animals in group (c) may be injected into others since some strains are apparently very slightly pathogenic for guinea pigs when freshly isolated but may remain viable in the tissues (blood and kidneys) and increase in virulence after passage through two or three animals (9, 10). Inoculate guinea pigs with cultures when these are obtained and animals inoculated with the original material show no evidence of infection. The golden hamster has been reported (11) as more susceptible than the guinea pig to infection with leptospirae especially *Lepto. canicola*, and may prove useful for isolation of these microorganisms.

Usually a guinea pig infected with a virulent strain of this species is intensely jaundiced and numerous petechial hemorrhages are found in the subcutaneous tissues. Hemorrhages are evident in practically all the tissues, but are especially prominent in those of the inguinal region and in the lungs where they have been called "butterfly hemorrhages."

Macerate the kidneys in salt solution, avoiding contamination as far as possible. Examine the urine and the kidney emulsion over dark-field illumination and use them for further cultural and animal tests if this is desirable. If no leptospirae are demonstrated, fix pieces of the lungs, liver, kidneys, and testicles in 10-per-cent neutral formalin so that they will be available for histologic examination.

BLOOD

If the patient is seen before the third day of illness, 10 ml. of blood should be collected in citrate with aseptic precautions. Centrifuge 5 ml. at 1500 r.p.m. from ten to fifteen minutes and examine the sediment by dark-field illumination. If leptospirae are found, confirm the result by animal test since motile filaments that may be mistaken for leptospirae are frequently present in material containing blood or other tissue elements (12). Identifications of leptospirae should never be based on the results of dark-field examination alone, unless the examiner is thoroughly familiar with these microorganisms.

If the blood is collected after the third day, remove the serum (see "General Instructions," p. 264), grind the clot in salt solution in a mortar, and make microscopic, cultural, and animal tests as described for urine. Centrifugation is not necessary if an anticoagulant has been employed.

Serologic Examination

Agglutination Test.—Combine in tubes approximately 11 mm. in diameter equal quantities (0.1–0.3 ml.) of actively motile cultures of leptospirae (preferably two strains of *Lepto. icterohaemorrhagiae* and one of *Lepto. canicola*) and serum undiluted and diluted 1:5, 1:10, 1:25, and 1:50 with salt solution. Mix by shaking and incubate for two hours at 35°–37°C. Examine moist preparations by dark-field illumination for agglutination. For control, test the culture at the same time with a known agglutinating serum and with that from a normal individual.

Loss of motility and definite clumping are significant even in a low dilution or in the undiluted serum since agglutinative properties have been demonstrated only in blood from individuals who have or have had leptospiral jaundice. High or rising titers after the fourteenth day of illness provide practically conclusive evidence of the disease.

CEREBROSPINAL FLUID

Centrifuge at 2000 r.p.m. for one-half hour and decant the supernatant. Examine the sediment as outlined in "Examination of Cerebrospinal Fluid," p. 520.

TISSUE

The demonstration of leptospirae in sections of the liver and kidney obtained at autopsy will establish or confirm a diagnosis of leptospiral jaundice.

WILD RATS AND OTHER ANIMALS

Since wild rats are known to be carriers of *Lepto. icterohaemorrhagiae*, these animals are occasionally received for examination from the locality in which cases of suspected leptospiral jaundice have occurred. If alive, anesthetize, bleed to death from the heart, and autopsy as directed for guinea pigs. Test the blood serum for agglutinative properties for *Lepto. icterohaemorrhagiae*.

Should other animals be received, follow the same procedure.

TREPONEMA RECURRENTIS (13)

Blood to be examined for the spirochete of relapsing fever should be collected at the height of the febrile reaction. Examine by dark-field illumination and also both thick and thin films stained by Giemsa's method as for malaria parasites (p. 34). *Treponema recurrentis* (*Treponema duttoni*) is either not present in the blood or is very few in number during the afebrile stage of the disease. If the spirochetes are not found by these procedures, mice may be inoculated and their blood examined (14, 15).

SPIRILLUM MINUS

BLOOD AND DISCHARGE

Animal Tests

Blood to be examined for the spirochete (*Spirillum minus*) of rat-bite fever (sokosho or sodoku) (14, 16–18) should be collected at the height of the febrile reaction and animals inoculated immediately. These microorganisms remain viable for only a short time after the blood has been drawn. Inoculate mice, white rats, or guinea pigs, or all three, subcutaneously or intraperitoneally. Examine the blood from each animal for spirochetes by dark-field illumination daily from the eighth to the fifteenth day after inoculation. If these microorganisms are found, the strain may be maintained by mouse passage. Stain films by the Giemsa (p. 34) or Wright method (p. 42) for permanent mounts.

Occasionally, *Sp. minus* can be demonstrated by dark-field examination or in stained films of blood from the human patient or from the fluid collected from a regional lymph node or from near the margin of a skin lesion. The spirochetes are so difficult to find, however, even at the height of the fever that the inoculation of animals is the procedure of choice.

A form of rat-bite fever is also incited by *Streptobacillus moniliformis* (see p. 331).

SPIROCHETES AND FUSIFORM BACILLI ASSOCIATED WITH VINCENT'S ANGINA

The type of fusiform bacilli and spirochetes associated with Vincent's angina may be present in large numbers in discharge from lesions in the mouth or throat or in membranes in diphtheria or streptococcus sore throat. They can also be demonstrated in exudate from bronchopulmonary spirochetosis and in fusospirochetosis of the genitalia as well as in indolent ulcers in various parts of the body. The finding of these spirochetes and fusiform bacilli in preparations from the mouth is of little diagnostic significance since they are usually in the discharge around the teeth in pyorrhea.

Since cases of diphtheria and streptococcus sore throat may be diagnosed as Vincent's angina, cultures of the discharge on coagulated-serum medium

(F15) as well as slide preparations should be examined. If both types of specimens are not furnished, the results of the examinations should be considered unsatisfactory.

DISCHARGE

Microscopic Examination

At least two slide preparations should be examined. Stain one with aqueous alcoholic crystal violet *(p. 37)* or dilute carbolfuchsin *(p. 43)*, the other by Gram's method *(p. 35)*. Examine for spirochetes and fusiform bacilli.

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CHAPTER 29

EXAMINATION FOR EVIDENCE OF RABIES AND CERTAIN OTHER VIRUS, RICKETTSIAL, AND MISCELLANEOUS INFECTIONS

RABIES

Rabies is an acute fatal disease of the central nervous system incited by a filterable virus (1, 2). It is most common in dogs but may occur in other animals such as cats and foxes; in fact, all warm blooded animals are believed to be susceptible. Man usually acquires the disease from the bite of a rabid dog. Other animals, however, may transmit the virus in a similar manner or it may gain access to the tissues through cuts or abrasions as a result of contamination with saliva or exudate. It has been claimed that rabies may be transmitted to animals by the bite of the vampire bat (British West Indies and South Africa) (3). Diagnosis is established by finding Negri bodies in the brain of the suspected animal or by inoculations of susceptible animals.

The period of communicability for man is not known, but for the dog it may be as early as four days before the onset of clinical symptoms and throughout the clinical course of the disease. The incubation period in man is usually from six to nine weeks, but may be as short as twelve days. In dogs, it is usually fourteen days or less. Since, however, this period is sometimes prolonged, an animal that has been bitten should be destroyed or isolated for at least four months. An animal that is apparently normal but that has bitten a person should not be killed, but kept under observation for one week. If it shows clinical symptoms of rabies, it should be killed at once and the head submitted for laboratory examination. Since Negri bodies can usually be demonstrated microscopically in the dog's brain but very little earlier than the appearance of clinical manifestations, it is important not to kill the animal before such symptoms are evident.

The animal should preferably be killed by gas or by a shot through the heart; strychnine or other chemical poisons may interfere with animal inoculation tests. The head should be kept cold, and, whenever possible, should be delivered by messenger. If messenger service is not available, the head should be placed in a container that closes tightly. This in turn should be put in a water-tight container and packed with cracked ice (Fig. 105). The use of dry ice is not recommended, since freezing the brain, which usually occurs, delays the examination and may affect the condition of the tissue.

The following information is desirable when animal heads are received for examination: data concerning the kind and breed of animal and whether it was in contact with other animals; information in regard to persons bitten or otherwise in contact with saliva; location and the character of injury; data concerning the use of fuming nitric acid to cauterize bites, or any other local treatment given; whether the animal died or was killed and if the latter, the means used for destroying it; whether the animal was confined and observed for an appropriate time before death, and if so, how long; symptoms of rabies, if any; history of vaccination against rabies or distemper.

BRAIN

Protect the hands with heavy rubber gloves and the eyes with goggles or a face shield. Hold the head firmly on the table and with a large section knife of the type used in autopsies make a median longitudinal incision through the skin, fascia, and muscles of the cranium to about the level of the eyes. Extend this incision laterally on both sides from a point slightly above the occipital protuberance, reflecting the soft tissue on both sides. Saw longitudinally through both pariteal bones, about one inch lateral to the midline. Saw transversely through the skull just above the eyes. Insert bone forceps through the anterior bone incision and lift off the top of the skull. If necessary, broaden the opening through the skull by breaking away the sides with bone forceps. Remove the dura and expose the cerebral hemispheres. See Fig. 41. With a scalpel or other suitable instrument, sever the nerves on the underside of the brain, cut the tentorium and the spinal cord, then remove the brain with as little laceration as possible. Place it in a suitable receptacle; for example, a paraffined paper plate. If the body of the animal is available and no typical Negri bodies are found in the stained films, make a general post-mortem examination in an attempt to determine the reason for the unusual behavior of the animal. Wrap the remains in paper and supervise their incineration. Wash the table with 10-per-cent formalin or a 1:1000 solution of bichloride of mercury. Sponge the gloves in the same kind of solution before removing from the hands, and then sterilize them with the instruments by boiling.

Macroscopic Examination

Describe the gross appearance of the brain, indicating especially the state of preservation, the presence or absence of adhesions and exudate, and whether the blood vessels are injected.

Microscopic Examination

Expose the hippocampus major (cornu ammonis) and remove a small cross-section about 1 mm. in thickness. Place it on a clean slide. Tease

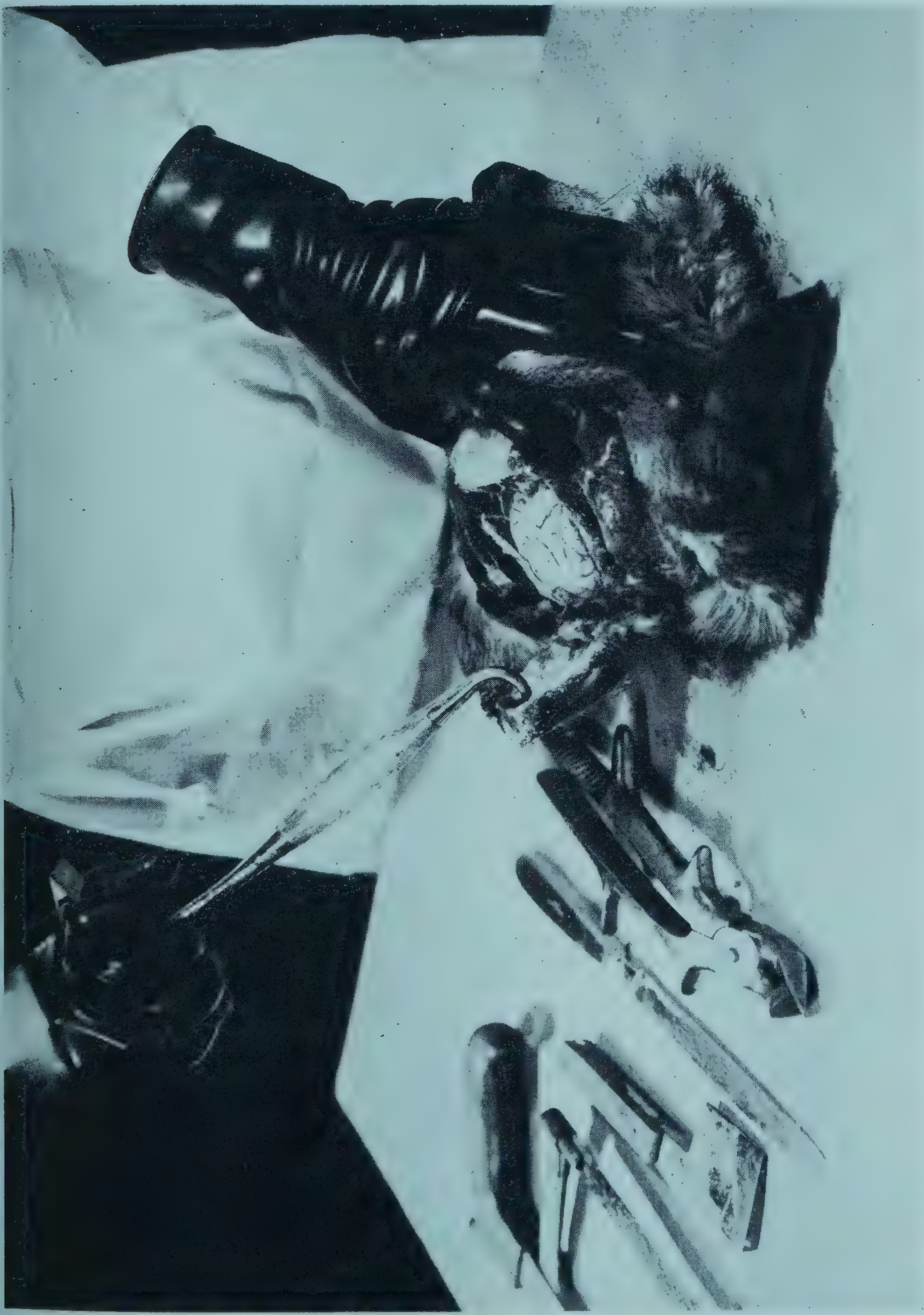

Fig. 41. Removal of Brain of Dog

away the white matter with a scalpel. With a clean slide used as a spreader, press evenly and firmly upon the tissue until it covers almost the width of the slide. Then draw the spreader toward the opposite end of the slide, while maintaining an even pressure. Discard the spreader in 10-per-cent formalin or a 1:1000 solution of bichloride of mercury, or sterilize it by boiling. Immediately plunge the film into Seller's stain (p. 40) and leave approximately five seconds. Wash in tap water, dry in air, and examine with the oil-immersion objective. Often, time may be saved by a study of the film under a low power, selecting areas containing numerous large ganglion cells.

The Negri bodies are stained cherry red. The basophilic or blue-colored masses in the bodies are nearly always clearly visible. The cytoplasm of the nerve cells stains more or less purplish blue, depending on the balance of the fuchsin and methylene blue; the nuclei and nucleoli of the nerve cells are more deeply blue. Stroma is rose pink; nerve fibers are a deeper pink. Neural sheaths do not stain. Bacteria, if present, are blue; muscle fibers, a brick red; red blood cells, a copper color.

Best results are obtained from the freshest tissue. As decomposition progresses, the histologic differentiation becomes more difficult.

Prepare films in the same manner from the cerebellum and cortex, if Negri bodies are not present in those from the hippocampus major.

When Negri bodies are not found by these procedures, stain films by a modification of Wilhite's method (4) (p. 42) and examine with the oil-immersion objective. In all cases in which typical Negri bodies are not found, make additional films from the other half of the brain and examine for at least one-half hour each not less than six to eight preparations.

Preparation of Paraffin Sections

When Negri bodies are not found, prepare stained sections from fixed tissue.

Rapid Method for Demonstrating Negri Bodies (Schleifstein) (5).—Cut blocks of fresh tissue not more than 3 mm. in thickness from the hippocampus major, the cerebral cortex, and the cerebellum. Fix in Zenker's solution, four hours at 37°C. Wash at least thirty minutes in running tap water. Dehydrate and clear as described for the dioxane-paraffin method for tissues (p. 541) except that the tissues to be examined for Negri bodies are left for only one hour in each of the following: dioxane, dioxane-paraffin mixture, and paraffin bath. Cut sections 4μ.

After the paraffin has been removed and the slides passed through the alcohols to water, place them on an electric plate; flood with freshly prepared dilute Wilhite's stain (p. 42) and gently steam, five minutes. Cool and wash quickly in tap water.

Decolorize and differentiate each section separately by gently swishing the slide in a jar of 90-per-cent alcohol until the section assumes a faint violet color. This is a particularly important step. Dehydrate, clear, and mount in neutral xylol-dammar (p. 548).

Negri bodies stain a deep magenta red and, under high magnification, the granular inclusions, dark blue. Nucleoli appear bluish black; cytoplasm, bluish violet; and red blood cells, copper color.

Goodpasture's Method (6).—Fix fresh tissue in Zenker's solution, twenty-four hours. Cut paraffin sections 4μ. Remove paraffin in the usual manner (p. 543). Treat with Goodpasture's stain (p. 35) ten minutes. Wash the sections in running water. Blot with filter paper and decolorize in 95-per-cent alcohol until the sections are pink. Wash in water and counterstain in alkaline methylene blue (p. 37), fifteen to sixty seconds. Wash in water. Dehydrate and decolorize for a few seconds in absolute alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Negri bodies are stained a sharp, bright red against a pale blue background. Inclusion bodies are blue; nucleoli, fibrin, and red blood corpuscles, red.

Animal Inoculation

If typical Negri bodies are not found in films or sections, inoculate white mice two to four weeks old with tissue from the brain (7, 8). Guinea pigs or rabbits may also be used but mice are preferable. Not only have mice been found to be highly susceptible to rabies virus, but the incubation period is from seven days to approximately three weeks; whereas in guinea pigs or rabbits it is from eleven days to three or four weeks.

Remove pieces of gray matter from the cortex, hippocampus, and cerebellum and place them in sterile 50-per-cent buffered glycerol (*F63.5*) (9), using three parts of the glycerol solution to one of tissue. The material from each region should be about 0.5 by 0.5 by 0.25 cm. in size. Whenever the brain is heavily contaminated, especially if decomposed, leave the material in glycerol for at least three days before using it for inoculation.

With aseptic technic, withdraw from the container strips of the glycerolated tissue. Remove the glycerol by rinsing in a few changes of salt solution and grind the strips in a mortar with a small amount of pulverized glass or alundum. Add 8 to 10 parts of salt solution and emulsify thoroughly. Allow the emulsion to settle and use the supernatant for inoculation.

Select three mice. Etherize lightly. Wet the skin over the skull with alcohol. Inject intracerebrally 0.02 to 0.03 ml. of the supernatant with a small syringe and a $\frac{3}{8}$ inch, 26-gauge needle. Introduce the needle through the skull at a point slightly to the left or right of the mid-line and just anterior to a line drawn from ear to ear.

Place together, in a jar or box, only mice inoculated with material from one specimen. Observe the animals daily for four weeks unless they die or are killed earlier. If more than one dies, inoculate three more. Chloroform one mouse between the seventh and tenth day after inoculation, since evidence of rabies, if present, can usually be demonstrated at this time. Death within four days of inoculation is usually not due to rabies and the cause should be determined, if possible. Chloroform mice that show symptoms of illness at any time during the observation period. Remove the brains and examine for Negri bodies. When the findings are inconclusive, inoculate more mice with the glycerolated material or with suspensions of brains of those that have died. Destroy all mice remaining well at the end of thirty days.

LYMPHOGRANULOMA VENEREUM

Lymphogranuloma venereum is a venereal disease of viral origin. It is fairly prevalent, particularly in the tropics. In the United States, the incidence is highest among negroes. No single laboratory procedure at present establishes a definite diagnosis, but several may contribute significant aid: the intracutaneous test of Frei (10, 11); a histologic study of the buboes (12); and a complement-fixation test of the blood, if satisfactory antigens are available (13). In the complement-fixation test, however, cross reactions have been reported with psittacosis, syphilis, meningopneumonitis, trachoma, virus pneumonia, and inclusion blennorrhea. Final diagnosis must, therefore, depend upon an evaluation of the history, clinical manifestations, and laboratory data (14).

Histologic study of the inguinal bubo is helpful in selected cases (14). Tissue is obtained by biopsy and paraffin sections made.

PSITTACOSIS (ORNITHOSIS)

The virus of psittacosis (ornithosis) is usually acquired through contact with diseased birds, although nurses have occasionally developed the disease while caring for patients. Since the outbreak in 1929–1930, few cases of psittacosis have been reported in New York State. The importation, breeding, sale, or offer of sale, of birds of the psittacine family has been prohibited. An exception, however, may be made with the approval of the State Commissioner of Health to permit their importation and breeding for scientific research or exhibition in public zoological gardens. Other types of birds in addition to those of the psittacine family, notably pigeons, have been shown to be infected with the virus.

Observe the precautions for handling highly infective material outlined in "General Bacteriologic Technic," p. 4. Confine the work to a room set aside exclusively for this purpose. Wear a gown and rubber gloves that are kept in the room; before entering, adjust goggles to protect the eyes and a sterile muslin mask over the nose and mouth.

SPUTUM

Examine slide preparations stained by Gram's method (p. 35) and Ziehl-Neelsen's method (p. 43) to determine the number and nature of microorganisms present. Perform any examinations that are indicated to be of possible significance in view of the microscopic findings and the clinical data.

Animal Tests

If relatively few microorganisms are present, inoculate two or three white mice intraperitoneally with 0.5–1 ml. of sputum. When many bacteria are present, add to 1 part of sputum, 2 parts of sulfonilamide solution (six 7-grain tablets in 100 ml. distilled water); use sulfathiazole, if staphylococci are indicated and sulfadiazine, if pneumococci are present (15). Mix by drawing in and expelling from a syringe. Incubate four hours. Centrifuge for five minutes at low speed and inject the supernatant into mice.

Keep the mice in glass jars placed in a pan containing 5-per-cent cresol compound or similar disinfectant. Cover the top of the jar with heavy wire mesh and cheesecloth to prevent dissemination of the virus in dust particles. (See Fig. 42.)

A wire mesh platform about 1½ inches high is placed in the bottom of the glass jar. This device permits mice to be kept for a week or more without being transferred. The wire mesh cover has a metal knob, about an inch high, in the center. A square of cheesecloth is placed over the cover and held tightly in place by a separate metal band slipped over the rim. The knob, by holding the cheesecloth off the cover, prevents the mice from gnawing at the cheesecloth. A water bottle is inserted through the cheesecloth and mesh cover.

Mice inoculated with the virus of psittacosis may show symptoms of infection within three or four days or may remain active for several weeks. Chloroform them when they are moribund or twelve days after inoculation if they are still alive. Immerse a jar containing dead mice in 5-per-cent cresol compound or similar disinfectant. When the carcass has been soaked, fasten with pushpins to a small board and open the body cavity. Prepare cultures and films from the heart's blood and from any lesions found in the organs. Exudate may be present and minute spots may be found on the liver; the spleen may be enlarged. Regardless of their appearance, always study the kidneys, spleen, and liver. Examine for bacteria. Also stain slide preparations by the Giemsa method (p. 34) or other procedures such as the methylene blue safranine stain (16) (p. 38) for the demonstration of psittacosis bodies (17, 18).

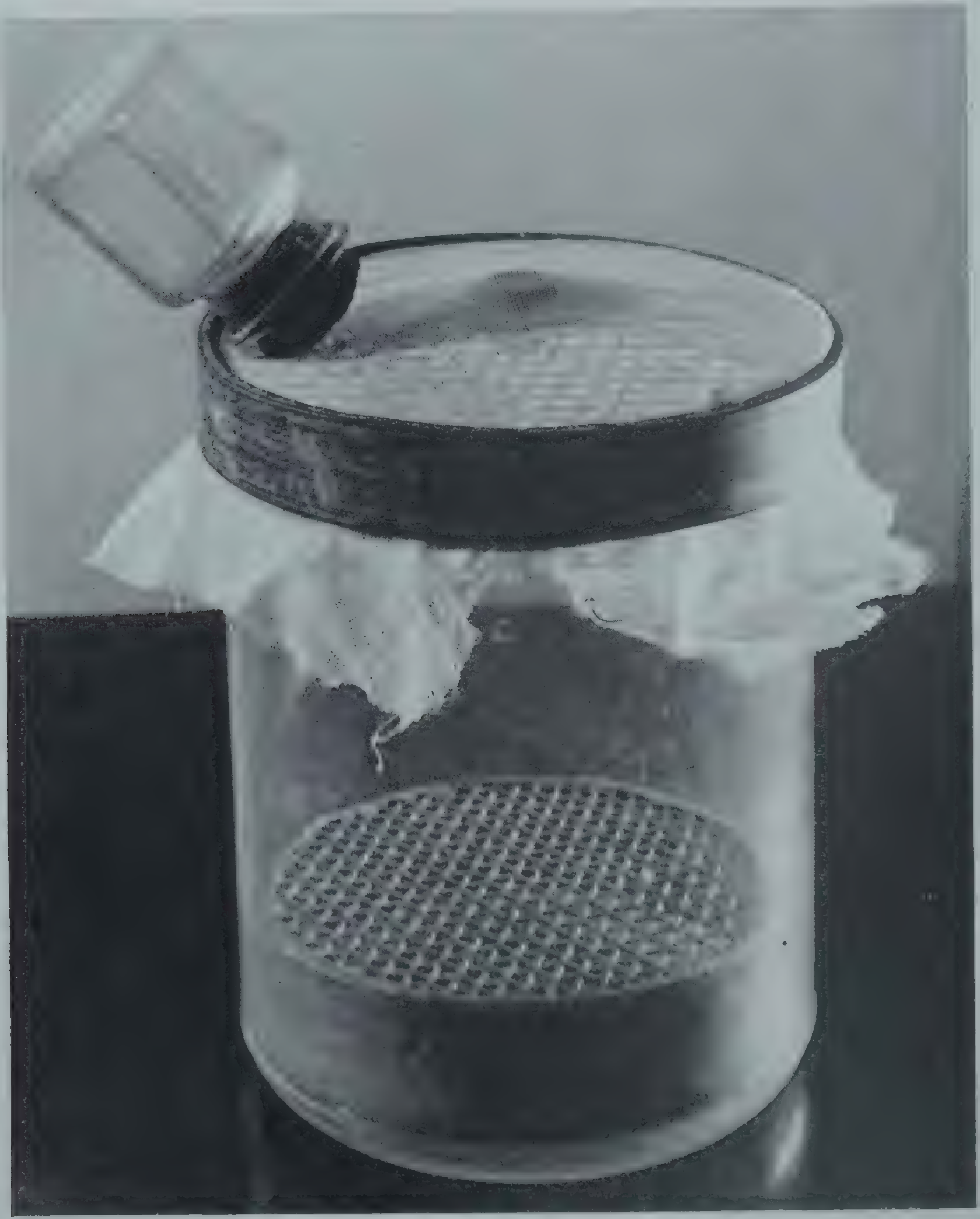

Fig. 42. Jar for Keeping Mice That Have Been Inoculated with Particularly Infective Material

Place portions of liver, spleen, kidneys, and any other organs with lesions in Zenker's solution for histologic examination and in phosphate buffered salt solution, pH 7.5 (*F68.5*) (15) so that they will be available for confirmatory study, if desirable. Freezing and drying *in vacuo* of material containing the incitant should permit satisfactory maintenance of strains for a considerable time. Meyer and his associates (19) have found that the virus in desiccated bird spleens remains virulent at 5°C. for two hundred and seventy-seven days.

If definite evidence of psittacosis (inclusion bodies in the reticuloendothelial cells) is found in films from the spleen, liver, or kidneys, and the histologic findings are characteristic, no further examinations are required. If psittacosis bodies are not demonstrated, or the results are not entirely conclusive, inoculate mice intraperitoneally with 0.5–1 ml. of emulsions of the tissues that were placed in buffered salt solution. Grind portions of the liver, spleen, and kidneys in a mortar with a few milliliters of salt solution. A small amount of sterile sand or alundum will facilitate grinding. Strain this suspension through gauze to remove particles of tissue that might plug the needle. The absence of psittacosis virus may be assumed only after passage through a series of at least three mice.

BLOOD

If antigen is available for the purpose, the complement-fixation test for evidence of psittacosis (20, 21) may be helpful in diagnosis, especially in case a specimen can be collected early during the course of the illness and another a week or two later. A marked increase in the strength of the reaction obtained with the second specimen suggests the presence of an infection. It must be kept in mind, however, that reactions may occur with antigen prepared from tissues containing the virus of psittacosis with specimens from patients with lymphogranuloma venereum (22, 23), and with atypical pneumonia (24–29). Some of the latter may be undiagnosed cases of ornithosis. Cross reactions have also been reported in specimens from human pneumonitis with virus from pneumonia in mice (30, 31).

BIRDS

When birds are to be examined for evidence of psittacosis, instructions for their submission should be given the physician or health officer. Living birds should never be shipped to a laboratory because of the danger to persons who would come in contact with them. They should be chloroformed, wrapped in muslin or cotton saturated with 5-per-cent cresol compound or similar disinfectant, placed in a water-tight receptacle such as a fruit jar, and packed in plenty of excelsior in a stout box for shipment. When received, soak the carcass in 5-per-cent cresol compound and fasten it with pushpins to a board of appropriate size; pluck the feathers from the ventral surface with forceps. Open the body cavity and examine as directed for mice. Examine the spleen, liver, and kidneys and any other tissues in which lesions are observed. If psittacosis bodies are not demonstrated or the histologic findings are not entirely conclusive, inoculate mice intraperitoneally with emulsions of tissue and proceed as directed for sputum. The study of the kidneys is of especial importance.

When a living bird is brought to the laboratory, chloroform it in a large can with a tightly fitting cover in which the cage may be placed. Then soak the dead bird and its cage in 5-per-cent cresol compound or similar disinfectant before making the autopsy.

SMALLPOX

Although clinical and epidemiologic data usually serve to differentiate smallpox or vaccinia from chickenpox, laboratory examinations may aid in the diagnosis. The procedure in present use is comparison of the cutaneous reactions induced in vaccinated and unvaccinated rabbits (32, 33) by saline suspensions of the specimen and of smallpox vaccine. Crusts or fluid from lesions should be so collected and handled that sufficient material relatively free from bacteria reaches the laboratory without exposure to high temperatures. A search for elementary bodies in film preparations from scrapings of skin lesions may also be helpful (34).

The results of animal tests are usually available within from four to seven days after inoculation, and require careful interpretation. For example, failure of a specimen to induce any visible reactions may be due to the absence of the virus of smallpox or vaccinia, or to inactivation during collection or handling of the specimen; cutaneous reactions due to bacterial contaminants may mask the typical reactions.

When fluid is received in a capillary tube, remove the wax from the end near the specimen with a needle. Break the wax seal from the other end with forceps. Attach a rubber bulb, expel the fluid into 0.5 ml. of salt solution in a tube, and wash the capillary tube by drawing up and expelling the solution several times. If fluid is received in more than one capillary tube, pool the washings in 0.5 ml. of salt solution.

When crusts from lesions are received, grind the material in a mortar and suspend in 1 ml. or less of salt solution.

With a 1-ml. syringe and a 26-gauge $\frac{3}{8}$-inch needle, mix the suspension of the specimen thoroughly by drawing in and expelling several times. Then inject 0.2 ml. intracutaneously on the back or side of first a normal rabbit and then of one vaccinated against smallpox from three weeks to two months previously. After all specimens have been injected, inoculate each rabbit with 0.5 ml. of a suspension consisting of one capillary of smallpox vaccine in 0.5 ml. of salt solution.

After completing the injections, make a cultural examination of each suspension (see p. 261).

Examine the rabbits daily and record the size, degree of redness, swelling, and induration, and presence of hemorrhagia and necrosis. If, after four days, no reactions have developed at the sites of injection of a specimen or the reactions correspond to those induced by smallpox vaccine, submit a report. Continue the observations for three or more days and, if any change in the reaction occurs, make a supplementary report.

By the film method (34), it may be possible to diagnose smallpox on the first or second day of the rash when it is in the papular stage. Experience, however, is required to distinguish the elementary bodies from those of chickenpox and to interpret the results.

INCLUSION BLENNORRHEA

The virus of inclusion blennorrhea usually attacks the conjunctiva of the newborn, but also produces conjunctivitis in adults (35–38). Diagnosis is best made by identification of the pathognomonic inclusion bodies present in the affected epithelial cells.

Specimens should be obtained by gently stroking the conjunctival surface of the eyelids with a small blunt curette. The material should be spread on slides and allowed to dry. If considerable exudate is present, films should also be prepared in the usual way to be examined for gonococci (see p. 282). Stain the former with Giemsa's or Wright's stain and examine for initial or elemental bodies. Use Giemsa's stain as directed for thin blood films (p. 34) and after twenty minutes in the staining solution, wash the slide first in water and then twice in 95-per-cent alcohol, five seconds each time (39). If Wright's stain is used (p. 42), add after exactly one minute, water to give approximately a 3- or 4-fold dilution. After five minutes, wash with water, drain, and blot dry (38).

GRANULOMA INGUINALE

Granuloma inguinale (granuloma venereum) is considered generally to be a venereal disease relatively prevalent in this country, especially among negroes. It is believed to be incited by so-called Donovan bodies (40), thought to be bacterial in nature (41, 42). They are oval-shaped Gram-negative microorganisms that resemble the Donovan bodies in kala azar and are present as inclusions in the large mononuclear endothelial cells. It is important to remember that syphilis, chancroid, or lymphogranuloma venereum may be coexistent.

Collection of Specimen

If the lesion appears to be clean, remove a bit of granulation tissue from the surface with a small bone curette or other suitable instrument (43). If, however, evidence of contamination exists, remove the surface exudate with a piece of sterile gauze moistened with salt solution and obtain the tissue from the deeper part of the lesion. When a biopsy specimen is desired, remove a portion of tissue from the margin of the lesion.

Examination of Films

Spread the tissue on a slide by firmly rubbing it over the surface of one or more slides or crush it between two slides and then spread along the slide. Allow to dry; then stain with tetrachrome (p. 41), and by the Giemsa (p. 34) or the Wright method (p. 42).

Histologic Examination

If significant findings are not obtained in the examination of films, a biopsy specimen may be desirable. Specially stained frozen sections have recently been recommended (44).

BARTONELLOSIS (45)

Bartonellosis, also known as Carrión's disease, occurs in man in the western portion of South America, especially in Peru, Ecuador, and Colombia. Two distinct types are recognized: oroya fever, the severe form, characterized by high fever and anemia; and verruga peruana, the cutaneous or chronic form, by nodular and papular eruptions. *Bartonella bacilliformis*, the incitant, is a minute Gram-negative rod-shaped or rounded microorganism, found in the red blood cells and in the reticulo-endothelial system. It may be demonstrated in blood films stained by Giemsa's method (p. 34), or on cultural examination in rabbit-serum semisolid agar (*F34E*) (46).

TYPHUS FEVER AND ROCKY MOUNTAIN SPOTTED FEVER (47, 48)

Both typhus fever and Rocky Mountain spotted fever are incited by *Rickettsia* and are transmitted by ecto-parasites. The procedures at present available for the demonstration and differentiation of rickettsia should be undertaken in a building so constructed as to insure strict isolation. Complement-fixation tests with rickettsial antigens have been shown to be of definite significance as an aid in the diagnosis of such infections (49–55) and are expected to supersede agglutination tests with *Proteus* X strains as soon as the antigens become more generally available.

Agglutination of the granular or somatic type with strains of *Proteus* X are usually of material diagnostic value. *Proteus* OX19 and sometimes *Proteus* OX2 as well, but not *Proteus* OX Kingsbury, are agglutinated in sera from patients having epidemic and murine typhus and Rocky Mountain spotted fever. On the other hand, agglutination with *Proteus* OX Kingsbury but not with *Proteus* OX19 or *Proteus* OX2 is reported to occur in blood from individuals having tsutsumagushi or scrub typhus. Therefore, tests with *Proteus* OX19 and *Proteus* OX Kingsbury should be performed for evidence of rickettsial infection. Agglutination may occur in relatively high dilutions of sera from some normal individuals. Reactions are significant only when they occur in a 1:640 or greater dilution or there is a definite rise in titer when a series of specimens collected at intervals of a few days are examined.

BLOOD

Serologic Examination

Macroscopic Tube-Agglutination Tests.—(See "General Instructions," p. 264.) Dilute the serum 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and test with alcohol-treated suspensions of *Proteus* X19, *Proteus* X2, and *Proteus* X Kingsbury (see preparation of bacterial suspensions, p. 802). For control, combine the suspensions with salt solution and with specific dilutions of sera known to agglutinate these strains of *Proteus*, preferably from patients with typhus or Rocky Mountain spotted fever. Incubate the tests for two hours at 35°–37°C., leave in the refrigerator overnight, and record the reactions. Centrifuge for ten minutes at approximately 2,000 r.p.m. and record the reactions again (see p. 265). If definite agglutination occurs in a 1:20 or greater dilution only after centrifuging, repeat the test, report the findings, and request another specimen. Otherwise, report the greatest dilution in which definite agglutination occurs before centrifuging.

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CHAPTER 30

SEROLOGIC TESTS FOR EVIDENCE OF SYPHILIS, TUBERCULOSIS, AND GONOCOCCUS INFECTION

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INTRODUCTION. The character and practical usefulness of serologic tests as an aid in the diagnosis of disease are advancing rapidly. Developments in the technic of complement fixation include the application of the test to infections other than syphilis, for example, to virus infections. The limitations of the test in bacterial infections have not yet been sufficiently defined and other aids to diagnosis are more generally practiced; in virus infections, while the technic has not yet, in most instances, been adequately developed, notable progress has nevertheless been made in the preparation and selection of the antigen. In virus diseases, as in syphilis, other aids to the early recognition of infection and to differential diagnosis are so inadequate that complement fixation, with further investigation, will undoubtedly prove of very real practical value.

In these circumstances, it is important to re-emphasize the use of quantitative procedures for evaluating the complement-fixation reaction, especially since the technic has a much broader scope, significance, and application than previous methods. Quantitative methods have been in routine use in this laboratory since 1937 (1). The procedures yield numerical values, the titers, that indicate the degree of reactivity, or the reagin content, of the serum, and that may be used by the clinician not alone as an aid to diagnosis but also in following the course of the disease and the effect of treatment. An important feature of the quantitative technic is that the degree of reaction over the range that is most difficult to evaluate in diagnosis—titers up to 10—is determined with reasonable accuracy. The physician is informed not only that a reaction has occurred, but also of the degree of the reaction. The determination of titers over 10, from 11 to 500 or higher, although thus far undertaken only in selected groups of cases, is proving very helpful in following the course of the disease during and after treatment, particularly as an aid in evaluating the new rapid methods of treatment that are now being extensively investigated. Not only has a sharper clinical characterization of the stage of the infection been possible in some instances, but the prompt retreatment of certain cases has been facilitated because a sharp reversal of titer has always accompanied and usually preceded clinical signs of relapse (2, 3, 3a).

The quantitative methods are practical adaptations of those used in experimental studies of the fundamental principles governing the reaction as it occurs with the different antigens (4–12). The technic as it is here described is similar in principle for syphilis, tuberculosis, and gonorrhea. The technical methods, in general, correspond and, hence, are described as a unit, differences being noted when necessary.

In the examination of specimens for evidence of syphilis, two preliminary tests (13–15) are used; these may be two complement-fixation tests with different antigens or one complement-fixation and one precipitation test.

This reduces the work with specimens that fail to react and permits quantitative examination of the other sera. In our experience with over 200,000 specimens a year, about 90 per cent do not react in the preliminary tests. They can be reported promptly; not more than 10 per cent thus require complete examination. The use of preliminary tests for tuberculosis or gonococcus infection cannot be recommended because significant changes in the reactivity of the serum with the antigens used in these tests frequently occur on standing.

A recent advance in serologic examinations for evidence of syphilis is the isolation from beef-heart extract of a hitherto unrecognized phospholipid, cardiolipin (16–20). Its identification as an essential antigenic constituent marks an important step forward in this field of investigation. For optimum reaction in the tests, cardiolipin requires adjustment with both lecithin and cholesterol (16, 21–23a). All three components are essentially chemically pure substances. Thus, an antigen of uniform composition and reactivity, except as the latter may be affected by properties peculiar to certain sera, is available and is now used in the routine complement-fixation test.

For thirty years the approval issued to the local laboratories in New York State for serologic tests for syphilis has been limited to complement fixation (24). The directors of the laboratories submit a description of the technic to be used; careful standardization, adequate controls, and satisfactory results are required for the issuance of the approval. Complement fixation has been considered the most sensitive and the most reliable method, especially since the precipitation tests, which have had an extended vogue, must, in the last analysis, be standardized on the basis of results obtained with carefully controlled complement-fixation procedures; and, for this purpose, the quantitative technic marks a most important advance. Although precipitation tests have not yet been approved, the precipitation reaction has been carefully studied with the expectation that reliable practical methods would be realized. There has been no restriction in the use of precipitation tests, provided the specimens have also been examined by approved complement-fixation procedures. At the present time the new cardiolipin antigen, composed of three essentially pure substances, offers exceptional opportunities for the investigation of discrepancies as well as for the analysis of low degrees of reactivity of questionable significance.

The practice of reporting the results of serologic tests in a manner that connotes a diagnosis has been avoided throughout the laboratory services. The clinician, not the laboratory, should assume full responsibility for the diagnosis. He may need to consult with the serologist in certain instances. A careful study of the 1936–37 and 1938 comparative series of tests conducted by the United States Public Health Service (1, 25) not only records the results obtained by different methods, including the quantitative technic, but also very strikingly illustrates the pitfalls of reporting results as "positive," "doubtful," or "negative."

PRINCIPLES GOVERNING THE QUANTITATIVE STANDARDIZATION OF REAGENTS IN THE COMPLEMENT-FIXATION TEST

The reactions that occur in complement-fixation tests are made manifest by different degrees of hemolysis, which reflect changes in the hemolytic activity of complement resulting from the presence of the patient's serum and antigen. In order to evaluate the reactions it is necessary to have a precise method for determining changes in complement activity. These changes can be determined most accurately by comparing the amounts of complement required for 50-per-cent hemolysis under different conditions. When this is done, the increments of complement so required are directly proportional to the increments of serum or of antigen tested, provided the serum or antigen is used in an amount to give the maximum reaction obtainable. Although a considerable excess of serum (antibody) fails to yield the maximum reaction of the serum, it does not diminish the reaction with a given amount of antigen but, beyond a certain point, excess of some antigens—for example, those used in the complement-fixation test for syphilis—yields lower reactions with a given amount of serum or, in other words, a prozone effect.

Because of these proportional relations between serum, antigen, and complement, the dose or doses of one of these reagents can be selected only in relation to those of the other two as well as in relation to the type of test that is to be used. If a quantitative evaluation of different degrees of reactivity in sera is desired, and prozones are to be avoided, the doses of serum, antigen, and complement must be adjusted to give the maximum reaction; also, multiple doses of serum, antigen, and complement must be used. The linear relationships between serum and complement, on the one hand, and between antigen and complement, on the other, simplify this adjustment. For example, if an amount of serum, 0.05 ml., reacts to a certain degree with a given number of complement units in the presence of the maximally reactive dose of antigen, 0.025 ml. and 0.0125 ml. of that serum will react to a similar degree with one-half and one-quarter the number of units of complement, respectively, and approximately one-half and one-quarter as much antigen will be required for maximum reaction. In the simplified quantitative test a single amount of serum is used with three amounts each of complement and antigen so adjusted as to approximate conditions for maximum reaction with sera having titers in the range up to 10. To determine titers above 10, dilutions of serum are tested.

To evaluate the results of complement-fixation tests, it is essential that the activity of the complement before and after exposure to serum and antigen be carefully determined; this can be done, as has been mentioned, by comparing the amounts of complement required under given conditions to give 50-per-cent hemolysis. It is evident from the sigmoid curve of hemolysis that the point of 50-per-cent hemolysis is a more accurate index of the activity of complement than points near complete hemolysis or complete inhibition of hemolysis, since a given change in complement in this region causes a relatively greater change in the degree of hemolysis obtained (figure 43). Changes in the hemolytic activity of complement, when slight, can be determined at other points of partial hemolysis, but when they are greater, determinations made at points on either side of 50-per-cent hemolysis are no longer uniform because of changes in the slope of the hemolytic curve that occur under these conditions. Furthermore, it is only when determinations under these conditions are made at 50-per-cent hemolysis that the linear relations between serum, antigen, and complement are observed and reactions can be quantitatively determined as in the procedure here described.

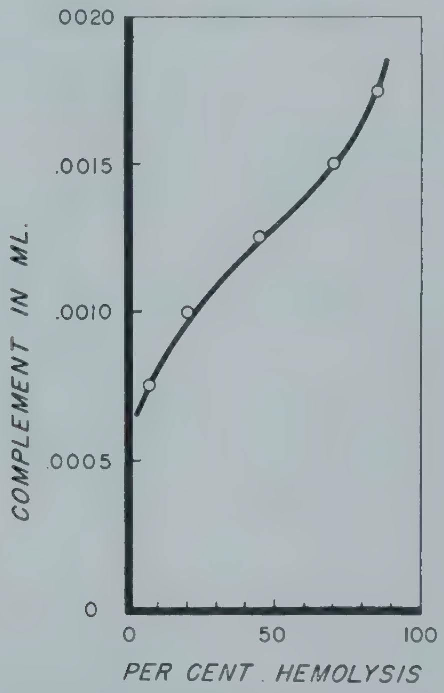

FIG. 43. COMPLEMENT TITRATION

Within given ranges of complement concentration, the change in slope is so slight as to be negligible; the relation between the amount of complement required for 50-per-cent hemolysis and the amounts required to give other degrees of hemolysis between 5 and 90 per cent is sufficiently uniform so that conversion factors expressing these relationships under different conditions can be determined. Such factors are derived from titrations of complement under the varying conditions of the test. By means of these factors the relative amounts of complement required for 50-per-cent hemolysis, as indicated by the degree of hemolysis obtained with a given amount under any condition, can be calculated and recorded. Thus, when some degree of hemolysis between 5 and 90 per cent occurs in a test with a known amount of complement, the relative amounts of complement that would be required under the same conditions to give 50-per-cent hemolysis can be read from previously prepared charts and need not be determined directly.

GENERAL DIRECTIONS

The total volume of all tests and titrations with blood serum is 0.5 ml. and with cerebrospinal fluid, 0.6 ml. The four reagents employed in the test—a suspension of sheep red blood cells, antisheep amboceptor, complement, and antigen—are standardized to conform to these amounts and are so diluted that quantities of 0.1 or 0.2 ml. may be pipetted in the test. Patient's serum is used undiluted in an amount of 0.05 ml. A change in the proportion of serum colloids in the test for syphilis may affect both the qualitative and quantitative determination of the reaction (5, 7, 26). Whenever serum is diluted for use in this test, as in the determination of titers over 10, the required dilutions are therefore prepared in freshly collected, pooled, nonreacting human serum and 0.05 ml. of each is used. Thus the concentration of serum constituents is uniform in all tubes except for the differences resulting from the several amounts of complement used. For convenience the terms "pooled nonreacting sera" and "normal sera" are used interchangeably in the text. For use with the bacterial antigens described, dilutions of patient's serum or of known immune sera that are used in standardization, or for purposes of control, may be made with salt solution. Salt solution, 0.85-per-cent C.P. sodium chloride, is used for preparing the suspension of sheep red blood cells, for making dilutions of amboceptor, complement, and antigen, and for equalizing volumes in the different tubes of the test or titrations. If necessary, as it has been with certain tubercle antigens, the physiologic salt solution may be buffered to a pH value of 7.6 by the addition of 10 per cent of Sörensen's borate buffer mixture (27) (*F67*).

Each of the reagents, except the cholesterolized antigen used in the complement-fixation test for syphilis, is kept in the cold room at from 3° to 6°C. when not in use; complement, since it may deteriorate rapidly, is kept in a pan of iced water while outside the cold room.

Separate pipettes should be used for each specimen and for each reagent. For measuring quantities of serum, antigen, or complement less than 0.1 ml., use a pipette of 0.2-ml. capacity graduated to 0.01 ml. For measuring

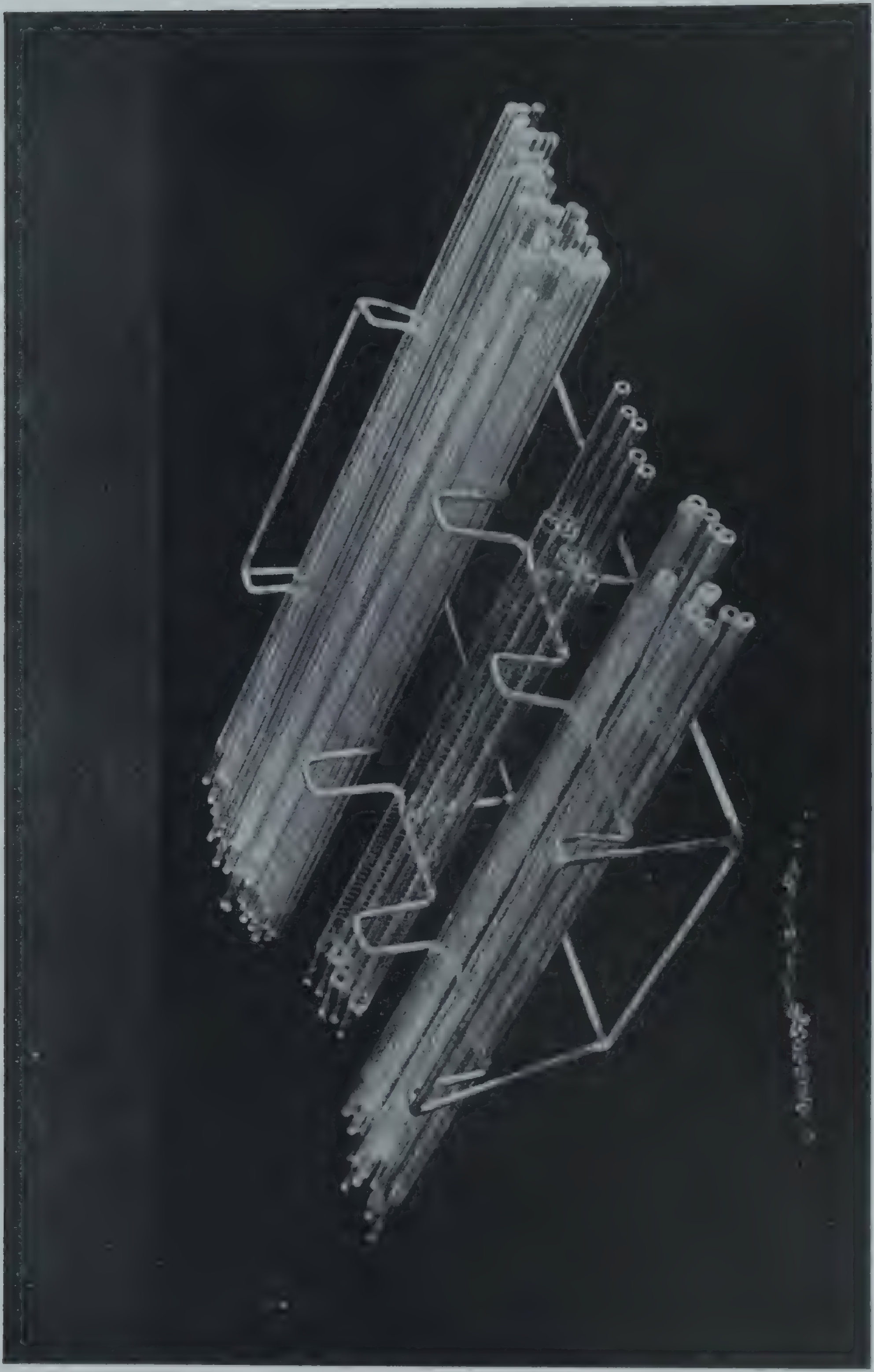

Fig. 44. Pipette Rack: A Convenient Device for Separating Different Types of Pipettes Used in Measuring Reagents

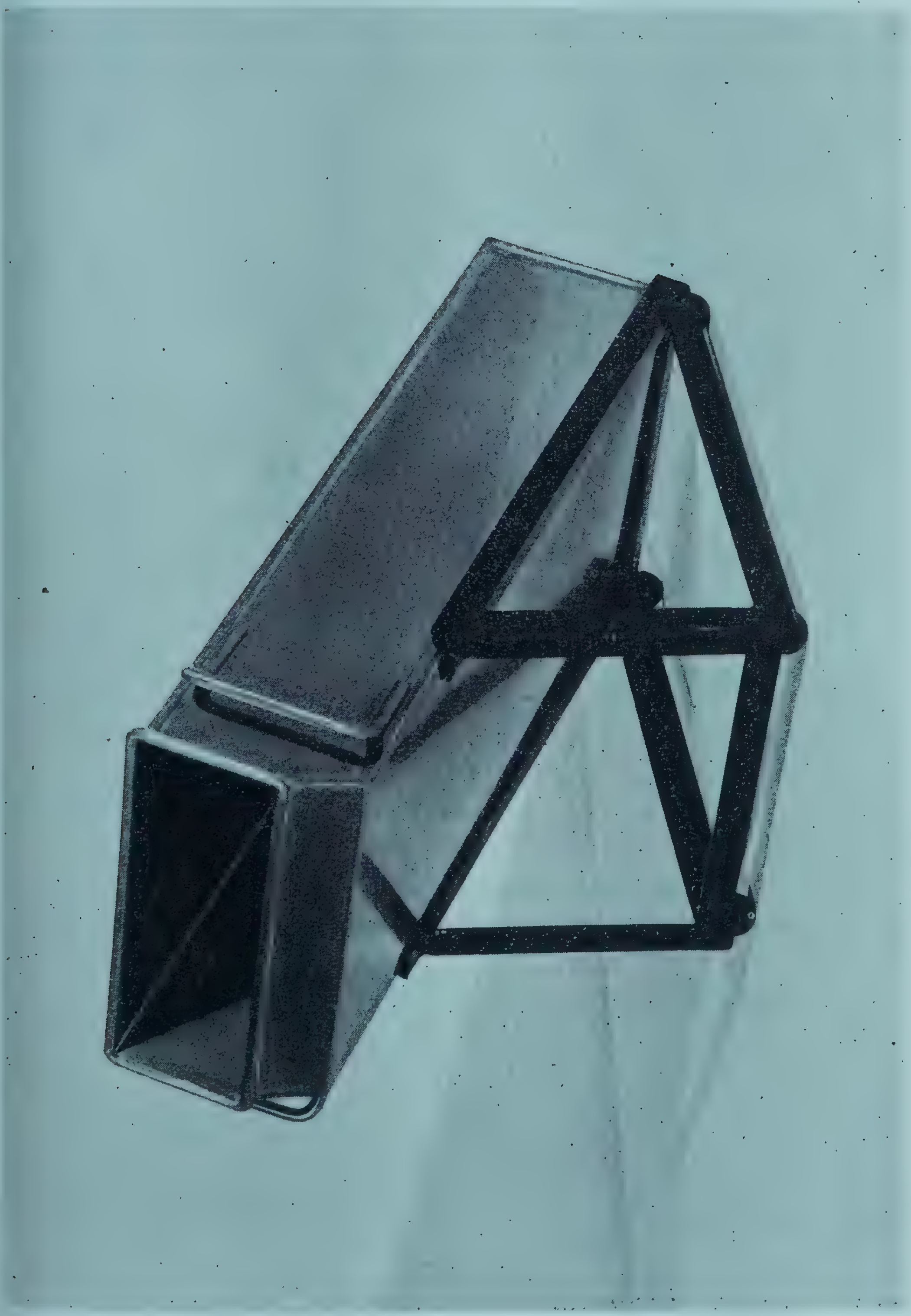

FIG. 45. CONTAINER FOR DISCARDED PIPETTES

Pipettes, immediately after use, are discarded into container partially filled with a suitable fluid, e.g., 1-per-cent sodium carbonate if pipettes are regarded as non-infective; otherwise, 1-per-cent cresol (*F75*). Complete immersion facilitates cleaning and reduces etching.

quantities of from 0.1 to 1 ml., use a pipette of 1-ml. capacity graduated to 0.1 ml. For quantities of from 1 to 10 ml., use a 2-, 5-, or 10-ml. pipette. For quantities greater than 10 ml., use a 10-ml. pipette or a suitably graduated cylinder. To prepare large volumes of diluted complement, use only accurately calibrated pipettes or flasks.

For measuring 0.1-ml. or 0.2-ml. quantities of reagents, pipetting machines have been found to provide increased accuracy and speed. The machines are electrically operated and are so adjusted that with each stroke of a piston exactly the desired quantity is delivered. The adjustment is checked each time the machine is used. See Fig. 46.

Do not take less than 0.1 ml. of fluid as the basis for any dilution.[1] When only a small amount of a high dilution is required, as in titrating amboceptor, make a 1:100 dilution and prepare from that the higher dilution desired. Mix the diluted reagent thoroughly by pouring back and forth from one flask to another at least three times or, if there are only a few milliliters of it, by drawing up in a clean 5- or 10-ml. pipette at least three times.

In titrations or tests for which the directions are given in tabular form, pipette the reagents in the order indicated beginning at the left. If the first reagent is to be measured in quantities of less than 0.1 ml., pipette it to the bottom of each tube. Pipette the other reagents toward the top, and shake the rack after the addition of each reagent. Add the salt solution in such a way as to wash down any reagents that may have adhered to the side of the tube. The tubes for the test should be of such size that the reagents can be readily mixed. Tubes 11- by 75-mm. are used.

Shake the suspension of sheep red blood cells thoroughly before withdrawing portions for preparing sensitized cells or for other purposes in order to obtain representative samples. During the water-bath incubation of titrations or tests to which the cells have been added, shake the racks sufficiently to keep the cells in suspension. Do not shake the racks so continuously or violently as to cause foaming of the liquid.

The period of incubation at 37°C. that is allowed for hemolysis varies in the different tests. Thus, in all the procedures of the tests for syphilis and gonorrhea, fifteen minutes are allowed, whereas in all of those for tuberculosis the time is sixty minutes. Similarly, the time and temperature of fixation vary: four hours in the refrigerator at from 3° to 6°C. is allowed in the test for syphilis whereas one and one-half hours in the water bath at 37°C. are more satisfactory with the bacterial antigens. For convenience in the descriptions of procedures that follow, these periods of primary

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[1] When only small amounts of patient's serum are available for use in end point titrations, dilutions may be made, if special care is taken, with amounts as small as 0.02 ml.

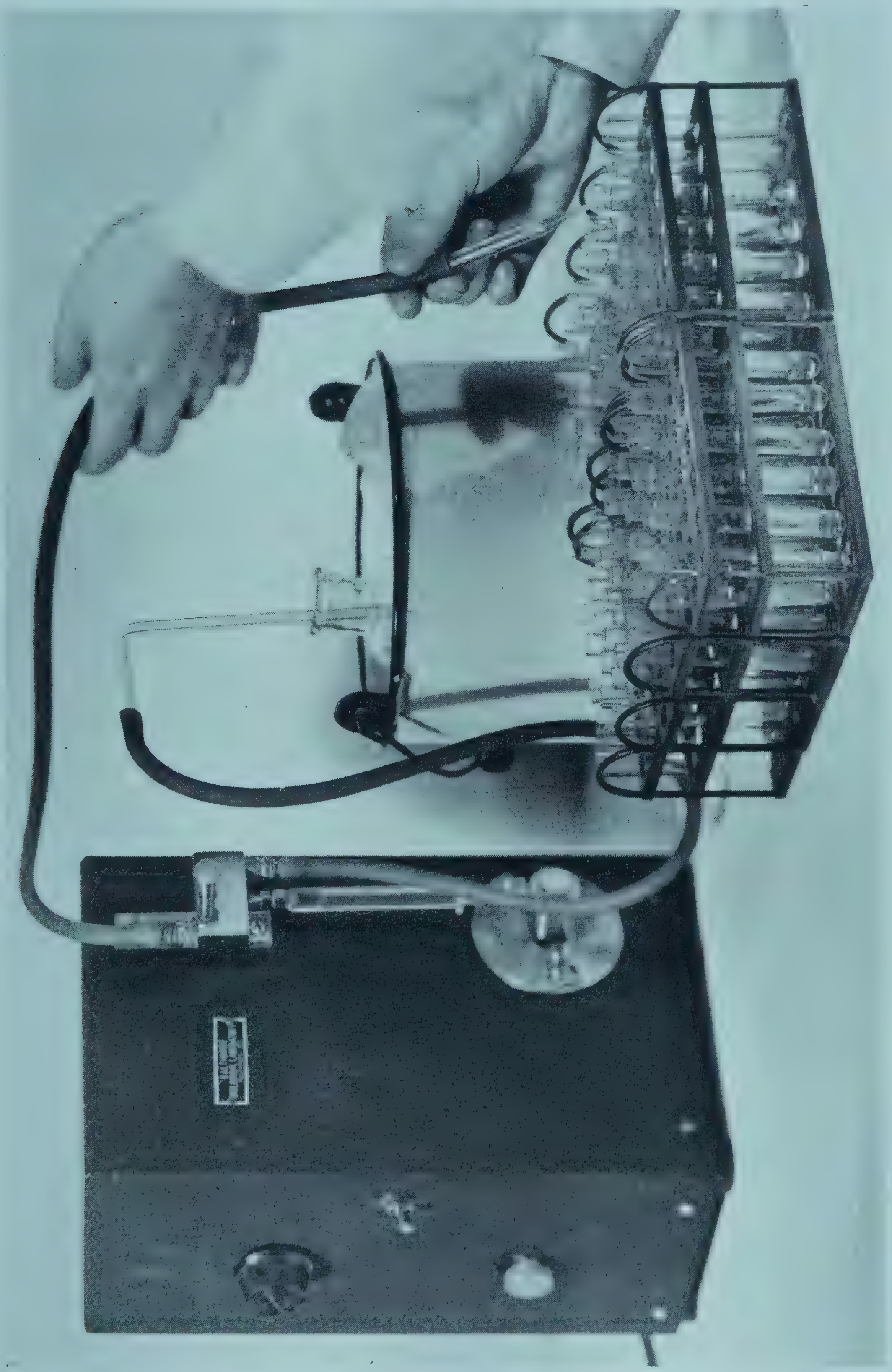

FIG. 46. GRAVITY VALVE TYPE OF PIPETTING MACHINE

fixation or of secondary incubation are indicated merely as "Standard for the Test" with a page reference to this statement. The temperature of the refrigerator or of the water bath in which tests are placed during the periods allowed for fixation or for hemolysis should not vary more than 1° above or below the temperature specified.

Record the degree of hemolysis obtained in the different tubes of tests or titrations and all necessary data concerning them, directly on the cards or forms designed for each. Readings are made by comparison with color standards.

COLOR STANDARDS

As an aid in the accurate reading of results of all tests and titrations, color standards (28), representing different degrees of hemolysis ranging from 0 to 100 per cent at intervals of 5 per cent, are used. The total volume in each corresponds to that of tests, namely, 0.5 ml. for standards used in reading tests of serum and 0.6 ml. for those used with spinal fluids. Known proportions of hemolyzed and unhemolyzed cells are used. Since the test dose of cells is 0.1 ml. of a 5-per-cent suspension, the proportions of hemolyzed and unhemolyzed cells used in the color standards conform to this amount. Thus, to represent 50-per-cent hemolysis, 0.05 ml. of a hemolyzed 5-per-cent suspension and 0.05 ml. of an unhemolyzed suspension would be used. The other reagents to be included in the standard depend upon the purpose for which it is to be used. It is impossible in routine practice to duplicate in the color standard the exact conditions of testing. For example, the test sera vary in color or appearance as do also the different dilutions of complement and antigen that are used. Standards to approximate the test conditions are prepared as described below. Since serum is not included, 5-per-cent hemolysis or less may be disregarded when obtained in tests with 0.05 ml. of serum. When a serum is hemolyzed and is to be tested, it is sometimes helpful to prepare a color control, corresponding to tube 1 of table 15 but containing 0.05 ml. of the hemolyzed serum and 0.15 instead of 0.2 ml. of salt solution. By use of this color control and by inspection of the sediment of cells in each tube of the test in comparison with the color standards, a fairly accurate estimate of the degree of hemolysis in the test, apart from the color contributed by the serum itself, can be made. One-tenth of a milliliter of inactivated 6-unit complement and 0.1 ml. of the dilution of antigen that is used in the test with 6 units of complement are included in color standards used for reading tests or titrations that contain these reagents. In the color standard for reading the complement titration, antigen is omitted and 0.2 ml. of inactivated complement diluted 1:40 is used in place of 6-unit complement.

To prepare the hemolyzed cells used in the color standard, lake in distilled water the sediment of cells from 10 ml. of the 5-per-cent suspension;

make isotonic with 8.5-per-cent salt solution and bring the total volume to 10 ml. Dilute a small amount of the laked cells 1:10 and prepare standards as indicated in table 15.

Centrifuge the standards representing 65-per-cent or less hemolysis and use the others with the cells in suspension.

TABLE 15

Preparation of color standards

TUBE	HEMOLYSIS	LAKED CELLS		SHEEP CELLS 5%	COMPLEMENT (INACTIVATED 6 UNITS)	ANTIGEN (DOSE USED WITH 6 UNITS OF COMPLEMENT)	SALT SOLUTION
		Undiluted	Diluted 1:10				
	%	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*
1	0	None	None	0.1	0.1	0.1	0.2
2	5	None	0.05	0.095	0.1	0.1	0.155
3	10	None	0.10	0.09	0.1	0.1	0.11
4	15	0.015	None	0.085	0.1	0.1	0.2
5	20	0.02	None	0.08	0.1	0.1	0.2
6	25	0.025	None	0.075	0.1	0.1	0.2
7	30	0.030	None	0.07	0.1	0.1	0.2
8	35	0.035	None	0.065	0.1	0.1	0.2
9	40	0.040	None	0.060	0.1	0.1	0.2
10	45	0.045	None	0.055	0.1	0.1	0.2
11	50	0.050	None	0.050	0.1	0.1	0.2
12	55	0.055	None	0.045	0.1	0.1	0.2
13	60	0.060	None	0.040	0.1	0.1	0.2
14	65	0.065	None	0.035	0.1	0.1	0.2
15	70	0.070	None	0.030	0.1	0.1	0.2
16	75	0.075	None	0.025	0.1	0.1	0.2
17	80	0.080	None	0.020	0.1	0.1	0.2
18	85	0.085	None	0.015	0.1	0.1	0.2
19	90	0.090	None	0.010	0.1	0.1	0.2
20	95	0.095	None	0.005	0.1	0.1	0.2
21	100	0.1	None	None	0.1	0.1	0.2

THE HEMOLYTIC SYSTEM

A 5-per-cent suspension of sheep red blood cells, rabbit antisheep cell amboceptor, and freshly collected guinea-pig serum are used. Amboceptor is used in an amount to provide maximum sensitization of the cells. Thus in the test of patient's serum the effect of any natural amboceptor that may be present is minimal. Complement is titrated daily to determine the unit value, namely, the amount required under standard conditions to give 50-per-cent hemolysis. While this amount is determined as accurately as possible, slight differences in the amount taken as the unit are relatively unimportant since the other dilutions of complement used in the test, namely, those containing 2, 3, 6, or 12 units, are all multiples of the dilution containing one unit and the result of the test is expressed as a ratio of the effect of serum and antigen upon complement to that of serum alone.

SUSPENSION OF SHEEP RED BLOOD CELLS

The suspension of red blood cells is prepared from defibrinated or citrated sheep blood collected not more than forty-eight hours previously.

In preliminary studies, sheep's blood collected aseptically in citrate glucose solution (28a, 28b) (*F72.5*) has been usable for periods up to four weeks. Washed cells prepared during the first few days following collection may show a somewhat increased resistance to hemolysis.

The sheep are selected by tests of trial bleedings. The cells should not hemolyze during washing or upon standing overnight in 5-per-cent suspension in salt solution; they should not be agglutinated in eighteen hours by the standard dilution of amboceptor nor, when sensitized, be unusually resistant or susceptible to hemolysis by complement. A fragility test may also be used as an aid in selecting sheep and to show alterations in cellular resistance due to bleeding or other causes. See p. 69.

If sheep are bled too frequently or too copiously, successive bleedings may become progressively unsatisfactory. There is also evidence that the maturity of the animal when regular bleeding is instituted affects the systemic response to bleeding. Lambs introduced into the bleeding schedule have exhibited more marked responses to bleeding than small but mature animals. As far as the record shows, once a sheep has been fitted into a schedule that is properly adjusted, bleeding may be continued indefinitely. One animal was bled at intervals of two weeks for fifteen years without any change in cells. Shortly before the animal died of old age, the cells showed a tendency to agglutinate in the presence of a relatively slight excess of antisheep amboceptor.

Filter defibrinated blood through neutral glass wool. Wash with at least five times the quantity of salt solution by repeated centrifugation for fifteen minutes (1400 r.p.m.). Counterbalance the tubes in the metal centrifuge cups each time. If the blood has been collected in citrate solution (*F72*), one part of blood in one part of 2.5-per-cent sodium citrate, place 15 ml. of blood in each 30-ml. centrifuge tube, add 0.85-per-cent salt solution to fill the tube, and mix by drawing up in a 50-ml. pipette, being careful to avoid injury to the cells. Centrifuge, remove the supernatant liquid, replace with fresh 0.85-per-cent salt solution, mix, and centrifuge as before. Repeat this process twice more, allowing fifteen minutes at about 1800 r.p.m. for the final centrifugation in order to pack the cells. Test the supernatant liquid for serum albumen with concentrated nitric acid—Heller's ring test. If any ring appears, wash th cells once more. After the final washing, the supernatant liquid should be clear and colorless; if the liquid is tinged with hemoglobin, do not use the cells.

The cells may be washed twice on the day before they are to be used. In this case, the salt solution should be drawn off after the washing, and the tubes closed with rubber stoppers and placed in the cold room.

If graduated tubes are used, read and record the volume of the packed cells in each before removing the supernatant salt solution of the final washing. If ungraduated tubes are used, carefully mark the level of the packed cells in each by pasting on a small label so that its upper edge coincides with the level of the packed cells. Draw off the salt solution and transfer all the cells to a graduate, using as little salt solution as possible for rinsing the tubes. Invert the tubes on filter paper to drain, and determine the quantity of packed cells that was contained in each by adding salt solution to each tube from a graduated pipette until the lowest point of the meniscus is on a level with the upper edge of the label. Note the quantity required and add a correction of 0.1 ml. for the approximate amount of moisture remaining in each tube after partial drying.

To prepare the 5-per-cent suspension, add sufficient salt solution to the washed cells to make the entire volume twenty times that of the packed cells.

To determine that the 5-per-cent suspension of sheep cells is satisfactory, dilute a 0.5-ml. quantity to 200 ml. with salt solution in a volumetric flask, mix well, and determine its turbidity in a candle turbidimeter (29). If the turbidity lies between 220 and 230, consider the suspension to be satisfactory. If the turbidity is above 230 adjust the suspension, using the following formula:

(Turbidity observed − 225):225::volume of salt solution to be added: original volume of 5-per-cent suspension prepared. If the turbidity is below 220 adjust the suspension as follows:

(225 − turbidity observed):turbidity observed::volume of washed packed cells to be added:original volume of washed packed cells used in preparing the suspension. Determine that the turbidity of the adjusted suspension is satisfactory before use.

The use of the photoelectric colorimeter should simplify the procedure and promote accuracy of the work (29a, 41a, 58).

To prepare sensitized cells, mix equal parts of the 5-per-cent suspension and the standard dilution of amboceptor. Pour the diluted amboceptor on the cells and pour back and forth from one flask to another at least three times to mix thoroughly.

ANTISHEEP AMBOCEPTOR

Production and Preparation

For the production of hemolytic amboceptor, rabbits weighing over 3000 grams are selected for immunization with sheep red blood cells. Those used previously for other types of work have been found suitable but they should be in good physical condition and have been rested for at least eight weeks.[2]

In our early experience, sera of satisfactory hemolytic activity were obtained when each injection of sheep red blood cells was followed by a dose of *Pasteurella lepiseptica*. For this purpose a strain of *Past. lepiseptica* known to contain heterophile antigen was selected (31). A salt-solution suspension of a 24-hour culture, killed by heating at 60°C. for thirty minutes, was used for the inoculum. Later this method was discontinued because of the high mortality among rabbits receiving the combined treatment.

Inoculum.—Filter through a cotton filter sheep's blood that has been collected in an equal part of 2.5-per-cent sodium citrate not more than forty-eight hours previously. For preparation of the filter, see "General Instructions," p. 622. With aseptic technic, wash in 0.85-per-cent salt solution by repeated centrifugation, according to the procedure described on p. 376.

The blood cells may be prepared either on the day of injection or the day before. After the final washing, draw off the supernatant salt solution and store the cells in the cold room.

Dosage.—All injections are made intravenously at semiweekly intervals, that is, on Tuesday and Friday, for two consecutive weeks. The volume of salt solution to be added to the packed cells is calculated so that the dose to be injected is contained in 5 ml. Give 0.125 ml. of cells in the first injection, 0.25 ml. in the second and third, and 0.375 ml. in the fourth. Inject very slowly and observe the rabbits closely for reactions. Should such occur, it may be advisable to omit part of the dose. Changes in schedule may be necessary depending upon the condition of the animal and the reactions induced.

Bleedings.—Three days after the fourth injection of cells, take a trial bleeding of 4 or 5 ml. from the marginal ear vein. Test the serum from the trial bleeding on the following day. If preliminary tests for hemolytic and agglutinative properties indicate that the serum may be satisfactory for use, bleed out the rabbit from the heart the following morning.

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[2] A large animal rather than a rabbit may be used for the production of amboceptor, since large amounts of this reagent are required. For a method of immunization see "Production of Anti-Sheep Amboceptor in a Mule," by Gilbert (30).

Tests of Trial Bleedings.—Inactivate the sera from trial bleedings, freed of blood cells, by heating at 56°C. for thirty minutes and prepare dilutions of 1:250, 1:500, 1:667, 1:1000, 1:1330, and 1:2000 in salt solution. Test for hemolytic activity as follows: Place 1 ml. of each serum dilution in a test tube. In a similar test tube place 1 ml. of 5-per-cent suspension of sheep red blood cells. Pour the amboceptor upon the cells and mix thoroughly by pouring back and forth at least three times. For each dilution of amboceptor prepare three tubes containing one, two, and three units of complement, respectively, in a total volume of 0.3 ml. Add 0.2 ml. of the cells sensitized with the dilution of amboceptor indicated. Place the mixtures in a water bath at 37°C. for fifteen minutes, and shake the tubes sufficiently often to keep the cells in suspension. Make readings against a color standard. Experience indicates that if the degrees of hemolysis are the same with the two lowest dilutions and decrease relatively slowly with succeeding dilutions, the serum will usually be satisfactory. In order to eliminate serum with marked agglutinative activity, test 1:100, 1:250, and 1:500 dilutions for agglutination with the same suspension of cells as was used in the tests for hemolytic activity, according to the procedure given under *Standardization* below.

Preparation and Distribution of Serum.—Draw off the serum from the clot, centrifuge to clarify, then inactivate by heating in a water bath at 56°C. for thirty minutes. Transfer to a sterile graduated cylinder, add an equal volume of C.P. glycerol, and mix thoroughly. Transfer the glycerolized serum to a bottle and store in the cold room. If two or more sera are prepared at the same time and in preliminary tests are found to have similar hemolytic and agglutinative activity, they may be pooled and standardized as a single lot.

Hemolytic amboceptor is dispensed in 5-ml. volumes. No sterility tests are made of the glycerolized material.

Standardization

To determine the agglutinating properties, add 0.1 ml. of different dilutions of the serum—1:100, 1:200, or higher if necessary—and 0.3 ml. of salt solution to tubes containing 0.1 ml. of a 5-per-cent suspension of sheep cells, and incubate for thirty minutes at 37°C. If no agglutination is evident at that time, make a second reading after the test has stood overnight in the cold room. Perform these tests with the cells of each sheep with which the amboceptor will be used. Discard any serum that causes agglutination in the dilution that proves optimum for use in complement-fixation tests.

To determine the optimum quantity of amboceptor for use in the test, titrate complement as indicated in table 16 using portions of a 5-per-cent suspension of cells sensitized with equal quantities of varying dilutions of the amboceptor outside the agglutinative range. To dilute complement 1:40 use not less than 0.5 ml. of undiluted pooled guinea-pig serum. In the titrations in which cells sensitized with the higher dilutions of amboceptor are used, it may be necessary to use larger amounts of complement than are indicated in table 16, namely, amounts increasing from 0.13 ml. to 0.2 ml. of the dilution to 1:40. Perform these titrations with the cells from each sheep with which the amboceptor will be used. On cross-section paper, plot the degrees of hemolysis observed against the quantities of complement tested in each titration and fit a smooth curve to the points by inspection as illustrated in figure 43. Read from the curves the amounts of complement required for 50-per-cent hemolysis in the different titrations and plot them against the quantities of amboceptor used as indicated in figure 47 which records the results obtained with cells from three different sheep in titrations of three pools of complement. Select as standard for use in tests, the dilution of amboceptor that contains an amount beyond which further increase fails to diminish appreciably the quantity of complement required for 50-per-cent hemolysis. For example, of the dilutions used in the titrations of figure 47, the 1:1000 dilution would be taken as optimum.

TABLE 16

Complement titration

TUBE NO.	COMPLEMENT (1:40)	SALT SOLUTION	SENSITIZED SHEEP CELLS	PERIOD FOR HEMOLYSIS
	ml.	*ml.*	*ml.*	
1	0.13	0.17	0.2	Standard for the test, see p. 372–374
2	0.12	0.18	0.2	
3	0.11	0.19	0.2	
4	0.10	0.20	0.2	
5	0.09	0.21	0.2	
6	0.08	0.22	0.2	
7	0.07	0.23	0.2	
8	0.06	0.24	0.2	
9	0.05	0.25	0.2	
10	0.04	0.26	0.2	
11	0.03	0.27	0.2	
12	0.02	0.28	0.2	

In titrations of complement with cells sensitized with the standard dilution of amboceptor, the relations of other degrees of partial hemolysis to that of 50-per-cent should not differ appreciably from those indicated by the conversion factors given in table 30 which were derived from titrations of complement alone; that is, without preliminary incubation and in the absence of serum and antigen. (See conversion factors corresponding to a $1/n$ value of 0.156 or 0.210.)

COMPLEMENT

Use for complement the pooled serum of at least three, preferably six or more, healthy guinea pigs, males or nonpregnant females, bled after twenty-four hours of fasting, on the day before the serum is to be used.

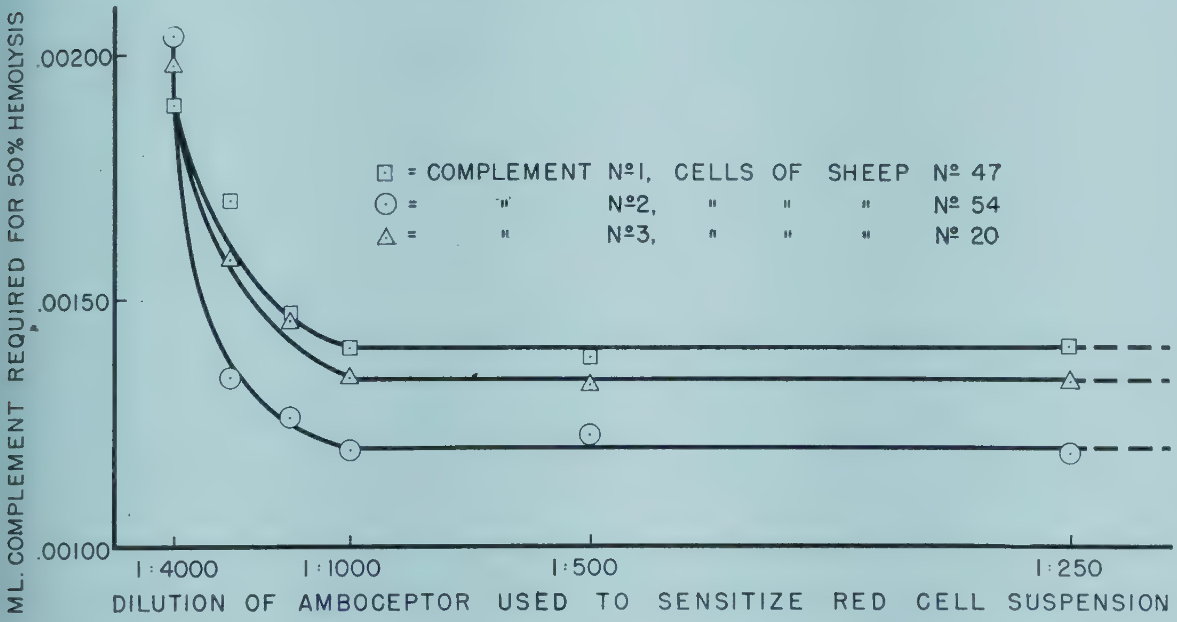

FIG. 47. STANDARDIZATION OF AMBOCEPTOR

Preserved complement has been reported to be satisfactory. If such preparations are used, their activity should be determined by accurate titrations with the reagents of the test.

Guinea pigs suffering from an infection of any sort may not yield serum satisfactory for complement, but the serum of animals used for experimental purposes, provided they have been rested two months and are in good condition, is usually satisfactory.

Used guinea pigs or small normal guinea pigs, 300–500 grams in weight, may be bled to death, but when large normal guinea pigs (500–800 grams) are available, 5–10 ml. of blood may be taken from the heart. In the former instance, avoid cutting the esophagus or trachea.

Collect the blood in sterile Petri plates and avoid disturbing it until it is firmly coagulated. Incubate at 37°C. for thirty minutes; rim each clot and put the blood in the cold room for one-half hour or longer. Pipette the serum from each specimen into a separate tube as the serum from individual guinea pigs is subjected to preliminary tests before being pooled.

Cover the tubes with loosely fitting metal caps. Centrifuge the serum for the minimum time necessary to deposit the cells—usually eight or nine minutes—withdraw the clear serum into clean tubes, and stopper the tubes before placing them in the cold room.

On the day the guinea pigs are bled, that is, the day before the serum is to be used for complement, the sera of the individual animals are subjected to preliminary tests. This is done to prevent the inclusion in the pooled complement of guinea-pig sera that are deficient or too potent in hemolytic activity, that contain excessive amounts of natural antisheep amboceptor, or that show, to a marked degree, the property of combining with antigen when no immune serum[3] is present. Sera found satisfactory for use are pooled and stored overnight in the refrigerator in a container packed in ice. The testing of individual sera for nonspecific fixability with antigen is of special importance as indicated in practice for many years (32–34). This is particularly true when only a few guinea pigs are bled; the presence in the complement pool of one serum possessing this property may result in a degree of nonspecific fixation that would affect the determination of the specific reaction (35).[4]

TABLE 17

Test of individual guinea-pig serum for hemolytic activity

TUBE NO.	GUINEA-PIG SERUM (1:10)	SALT SOLUTION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
	ml.	*ml.*	*ml.*	
1	0.04	0.26	0.2	Standard for the test,
2	0.01	0.29	0.2	see p. 372–374

Test for Hemolytic Activity

Test each guinea-pig serum for hemolytic activity as indicated in table 17. Record the hemolysis with each amount of serum. Include in the pooled complement only the guinea-pig sera that give 95- to 100-per-cent hemolysis in the larger amount tested. The test with the smaller amount serves in rare instances as a guard against including in the pooled complement too large a proportion of guinea-pig sera of unusually marked hemolytic activity, for example, sera giving 75-per-cent or more hemolysis.

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[3] Immune serum is used in a broad sense to mean all sera reacting specifically with antigen.

[4] Harris and Portnoy (21) report that guinea-pig sera do not react with the cardiolipin antigen. Our experience thus far confirms their observation but for the time being the tests with this antigen are being continued.

Test for Natural Antisheep Amboceptor

Since the sheep cells that are used in the complement-fixation test are maximally sensitized with rabbit antisheep cell amboceptor, the effect of any natural amboceptor in guinea-pig serum is probably minimal. Nevertheless, it has seemed desirable to exclude from use guinea-pig sera having more than slight amounts of natural amboceptor until more information regarding the action of such antibodies is available.

Test each guinea-pig serum for natural antisheep amboceptor, including with each group of tests a control of the cells suspension, as indicated in table 18.

Centrifuge each tube to deposit the cells, and record the degree of hemolysis in each.

TABLE 18

Test of individual guinea-pig serum for natural antisheep amboceptor

TUBE NO.	GUINEA-PIG SERUM (1:10)	SHEEP CELLS (5 PER CENT)	SALT SOLUTION	PERIOD FOR HEMOLYSIS
	ml.	*ml.*	*ml.*	
1	0.1	0.1	0.3	Standard for the test,
2	None	0.1	0.4	see p. 372–374

The hemolysis is due to natural antisheep amboceptor in the guinea-pig serum, provided the cells control shows no hemolysis.

Include in the pooled complement only guinea-pig sera containing no more than a very slight amount of natural amboceptor according to this test, namely, sera giving 5-per-cent or less hemolysis.

Test for Nonspecific Fixability

Test each guinea-pig serum for nonspecific fixability as indicated in table 19.

Record the time required for complete hemolysis with each guinea-pig serum. If hemolysis is not complete at the end of ten minutes, record the degree of hemolysis observed.

Include in the pooled complement only the sera with which 95- to 100-per-cent hemolysis occurs in ten minutes or less.

Titration of Pooled Complement

On the day the complement is to be used, dilute 1:40 two samples of the pooled serum using not less than 0.5 ml., and titrate the hemolytic activity as shown in table 16.

In the complement titration, use sensitized cells prepared from portions of the 5-per-cent sheep-cell suspension and the standard dilution of amboceptor prepared for the day's work.

Prepare the sensitized cells required for complement-fixation tests from the diluted amboceptor remaining, not more than ten or fifteen minutes before it is time to add them to the first set of tests.

Record the degree of hemolysis in each tube.

On cross-section paper, plot the degrees of hemolysis observed against the amounts of complement tested (milliliters of undiluted guinea-pig serum) and, by inspection, fit a smooth curve to these points. As mentioned previously, in titrations of complement with cells sensitized with the standard dilution of amboceptor the relations of other degrees of partial hemolysis to that of 50 per cent should not differ appreciably from those indicated by the conversion factors in table 30, p. 416, which were derived from titrations of complement alone; that is, without preliminary incubation and in the absence of serum and antigen. (See in table 30 conversion factors corresponding to a $1/n$ value of 0.156 for titrations incubated for fifteen minutes at 37°C. or of 0.210 for titrations incubated for one hour at 37°C.) Thus, it is helpful in fitting a curve to the observed points to draw also the so-called "standard" curve reconstituted from the conversion factors. To do this, estimate by inspection of the plotted observed points the amount of complement required for 50-per-cent hemolysis. Multiply this amount by the conversion factors corresponding to the different degrees of hemolysis from 5 to 90 per cent. Plot the amounts so obtained against the degrees of hemolysis from which they were derived and fit a

TABLE 19

Test of individual guinea-pig serum for nonspecific fixability

TUBE NO.	ANTIGEN (DILUTION IN USE)	GUINEA-PIG SERUM (1:10)	SALT SOLUTION	FIXATION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
	ml.	*ml.*	*ml.*		*ml.*	
1	0.2	0.05	0.05	Standard for the test,* see p. 372–374	0.2	10 min. at 37°C.

* In the complement-fixation tests for syphilis, the period for fixation is four hours at from 3° to 6°C., but in these preliminary tests, the complement and antigen are exposed for only two hours at from 3° to 6°C., it having been found that guinea-pig sera which are markedly affected by exposure with the antigen for four hours are usually perceptibly affected in the shorter period. Also, as a result of experience, only the dilution found optimum for use with weakly reacting syphilitic serum need be used.

curve to the points.[5] When the unit amount of complement has been chosen correctly the observed points in the titration will be uniformly distributed in relation to the standard curve. The unit amount should usually range from about 0.0013 to 0.0017 ml. in tests allowing fifteen minutes for hemolysis, and from about 0.0007 to 0.0011 ml. in tests allowing sixty minutes for hemolysis.

The results of tests may be less accurate when the quantity of complement giving 50-per-cent hemolysis appreciably exceeds these limits. When it is small, deterioration may be marked in tests incubated at 37°C., for example, in the preliminary complement-fixation tests for syphilis, and numerous reactions that are not significant may occur, necessitating either repetition of the tests or the examination of an undue number of specimens in the quantitative procedure. When the amount is unusually large, on the other hand, there may be danger of missing reactions of low degree because of the activating effect of patient's serum upon such complement. Therefore, when pools of greater or of less than average activity are used, it is the practice to adjust the quantity of complement giving 50-per-cent hemolysis before using it as a basis for preparing the dilutions of complement to be used in tests. Experience indicates that the amount of complement giving 50-per-cent hemolysis in titrations incubated for fifteen minutes at 37°C. after the addition of sensitized cells may be reduced by 5 per cent when it is only slightly greater than 0.0017 ml., or by 10 per cent when it approaches 0.002 ml. When the amount exceeds 0.002 ml., the complement is discarded and a fresh pool obtained. On the other hand, when the quantity required for 50-per-cent hemolysis is as small as 0.0012 ml., it is increased by 3 per cent, or if as little as 0.001 ml., by 5 per cent. Complement more active than this has not been encountered.

Similarly, reductions of from 5 to 10 per cent are made in the amount of complement required for 50-per-cent hemolysis in titrations incubated for sixty minutes following the addition of sensitized cells, whenever it exceeds 0.00125 ml.; adjustment to increase the amount has not been necessary.

The quantitative test, since the result is expressed as a ratio, is not greatly affected by these variations in complement activity. However, the ratio tables, tables 35–46, that give the titers of reactions occurring with different amounts of complement are based on determinations made with complement of average activity and might require a small correction to allow for changes in slope of the hemolytic curves that would be obtained with complement of definitely greater or less than average activity.

The amount of complement designated as a unit for use in any complement-fixation test should correspond approximately to the amount that would give 50-per-cent hemolysis under the conditions of the test. The titers of sera in different tests are then comparable since they represent corresponding changes in the unit of complement. The unit amount need not, and in fact cannot, be determined exactly because the effect of the time and temperature of fixation upon the hemolytic activity of complement may vary with every serum tested. It is important, however, that the different dilutions of complement used in tests be exact multiples of the amount taken as one unit.

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[5] For convenience, tables can be prepared containing the values necessary for plotting the so-called "standard" curves of titrations in which the amounts of complement required for 50-per-cent hemolysis vary within the limits considered satisfactory for use in tests, namely, from 0.001 to 0.002 ml. in the tests for evidence of syphilis or gonococcus infection or 0.0006 to 0.0012 in the test for evidence of tuberculosis.

Because fixation for four hours at from 3° to 6°C. has little or no effect upon the hemolytic activity of complement, the amount of complement required for 50-per-cent hemolysis in the titration just described can be taken as one unit for use in the complement-fixation test for syphilis. In the tests with the tubercle and gonococcus antigens that require fixation for one and one-half hours at 37°C., a larger amount is necessary because of the greater deterioration of complement at this temperature. In our experience, an amount 1.5 times that required for 50-per-cent hemolysis approximates satisfactorily the unit activity of complement under the conditions of these tests. This amount is considered as one unit in calculating the amounts of complement to be included in the different dilutions used.

Multiply the number of milliliters of complement representing 1 unit by 12 to determine the amount of guinea-pig serum to make a dilution containing 12 units in 0.1 ml., and calculate the amount of salt solution required to dilute to that volume by subtracting the product from 0.1 ml. Prepare a quantity of a mixture in this proportion sufficient for the day's tests and for the preparation of further complement dilutions needed.

After five or ten minutes, prepare additional dilutions to contain 6, 3, 2, and 1 unit by adding to one part of the dilution containing 12 units one, three, five, and eleven parts, respectively, of salt solution. For the test with gonococcus antigens a greater number of doses of complement is used; namely, 1, 1.5, 2, 3, 4, 6, 8, and 12 units. It is convenient to prepare dilutions containing 8, 4, 2, and 1 unit by adding to one part of the dilution containing 12 units, five-tenths, two, five, and eleven parts respectively of salt solution and to use additional amounts of these dilutions equivalent to 1.5, 3, and 6 units in the tests.

Check the dilutions, as indicated in table 20. If the dilutions have been correctly prepared, each test of a given set of dilutions will contain the same amount of complement and should, therefore, give the same result. The degree of reaction with dilutions prepared for use in the test for syphilis and gonococcus infection may vary on different days between 70- and 85-per-cent hemolysis and, with those for the test for tuberculosis, between 30- and 45-per-cent but the results on a given day should not differ by more than 10-per-cent hemolysis. If greater variation occurs, repeat the tests and, if there is evidence of error in dilution, prepare fresh dilutions and check them in the manner just described. When additional dilutions are needed, prepare and check them in a similar way.

TABLE 20

Test for accuracy of complement dilution

TUBE NO.	AMOUNT OF COMPLEMENT IN 0.1 ML.	AMOUNT OF DILUTION USED	SALT SOLUTION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
Complement-fixation test for syphilis					
	units	*ml.*	*ml.*	*ml.*	
1	6	0.02	0.28	0.2	15 minutes in the water bath at 37°C.
2	3	0.04	0.26	0.2	
3	2	0.06	0.24	0.2	
4	1	0.12	0.18	0.2	
Complement-fixation test for tuberculosis					
1	6	0.01	0.29	0.2	60 minutes in the water bath at 37°C.
2	3	0.02	0.28	0.2	
3	2	0.03	0.27	0.2	
4	1	0.06	0.24	0.2	
Complement-fixation test for gonococcus infection					
1	8	0.01	0.29	0.2	15 minutes in the water bath at 37°C.
2	4	0.02	0.28	0.2	
3	2	0.04	0.26	0.2	
4	1	0.08	0.22	0.2	

PREPARATION OF ANTIGENS

TEST FOR EVIDENCE OF SYPHILIS

The cardiolipin-lecithin-cholesterol antigen is now used in complement-fixation tests of all specimens. The cholesterolized alcoholic extract of beef-heart tissue previously employed is used when a test with a second antigen appears desirable. Both antigens are dispensed in amounts of 25 ml. in amber glass bottles with screw tops lined with tin foil.

Preparation of Cardiolipin and Lecithin (16–20, 36)

Cardiolipin is a phospholipid. It contains no nitrogen. It is an acid, having one acidic valence for each phosphorus atom; in the free acid form it is unstable, and it is used in the form of the sodium salt, which is very stable under proper conditions of storage. The isolation and purification of cardiolipin depend on the preparation of salts of varying solubility in organic solvents, the barium and cadmium salts being the most useful. In crude tissue extracts, such as have been widely used as antigens, cardiolipin probably is present as either a potassium or sodium salt.

While cardiolipin probably occurs in other tissues, it has so far been prepared in quantity only from beef heart, which is also a convenient source of lecithin for use in the antigen mixture.

Both cardiolipin and lecithin contain unsaturated fatty acids and must therefore be handled with precautions against oxidation. The following general principles and technics are observed throughout the preparation unless exceptions are specifically noted.

Use of carbon dioxide to displace air.—In all vacuum distillations, CO_2 from a Kipp generator is admitted through the capillary instead of air. If the procedure requires evaporation to a small volume or to dryness, CO_2 is admitted instead of air when the vacuum is released. For vacuum distillation a water-pump is used.

Drying.—Wherever possible, the operative procedures have been so devised that the lipids are handled in solution, without drying. At some points this is not practical and directions are given to evaporate to dryness *in vacuo*. In such cases the next solvent to be used is added without delay, as soon as the flask is opened. When solutions in volatile solvents, such as ether, are transferred, the rinsing of glassware is carried out promptly so as to avoid the formation of dry lipid films and their exposure to air.

Light.—All lipid fractions are protected from light whenever practical and are never exposed to direct sunlight.

Temperature.—The available evidence indicates that cardiolipin and lecithin may be stored for some time at room temperature without damage; however, it is a good routine practice to store all solutions at ice-box temperature (3°–6°C.) when not in use. Solutions of cardiolipin in absolute alcohol, stored in the cold, have remained chemically unchanged for periods up to two and one-half years.

Solvents.—All solvents used in the preparation should be of C.P. grade, except for the preliminary extractions where U.S.P. solvents have been found satisfactory. Ether should be of C.P. anhydrous quality.

Distilled water is always used as a lubricant for stoppers and stopcocks of separatory funnels instead of stopcock grease.

Reagents.—Barium chloride ($BaCl_2$): 200 grams in 1 liter of water.

Cadmium chloride ($CdCl_2$): (1) Dissolve the salt in its own weight of water. (2) Dilute solution (1) with an equal volume of H_2O; this more dilute solution is convenient if small lots of material are to be precipitated and is also used in testing small samples for completeness of precipitation.

Sodium sulfate (Na_2SO_4): 50 grams in 1 liter of H_2O.

Sodium chloride ($NaCl$): (1) Saturated solution: about 360 grams per liter of H_2O. (2) Solution (1) diluted with an equal volume of H_2O.

Hydrochloric acid (HCl): 10 ml. concentrated HCl diluted to 100 with H_2O.

Ammonia (NH_3) in methyl alcohol: From a tank of compressed NH_3 gas introduce NH_3 into methyl alcohol which is kept chilled in ice. Weigh the mixture occasionally until the increase in weight is about 25 per cent of the original weight of the solvent.

Saturated barium hydroxide ($Ba(OH)_2$): Heat 6 grams of $Ba(OH)_2 \cdot 8\ H_2O$ to boiling with 100 ml. of H_2O. Cover and allow to settle; decant while warm into a stock bottle. Do not attempt to filter. The sediment of $BaCO_3$ settles readily on standing and the clear solution is pipetted off when required.

Preparation and Extraction of Tissue.—Remove fat and connective tissue from fresh beef hearts and grind in meat chopper. Weigh 1-kg. portions into 3-liter Erlenmeyer flasks and to each kilogram add 1200 ml. of U.S.P. acetone. Stopper the flasks, shake vigorously, and let stand overnight at room temperature. Filter off acetone by suction, return tissue to flasks, and re-extract for twenty-four hours with 1200 ml. of acetone per kg. Filter off the second acetone extract. Dry the tissue before a fan and grind it to a fine powder. Weigh 300-gram portions of tissue powder into 3-liter Erlenmeyer flasks and add 2 liters of methyl alcohol (U.S.P. 95 per cent) for each 300 grams. Shake vigorously. Let stand at room temperature, protected from light, with frequent shaking. After from 5 to 7 days, filter by suction and re-extract with methyl alcohol in the same manner. Repeat, making two extractions altogether. When the second extract is filtered off, wash the tissue on the filter as follows: Disconnect the suction, pour about 400 ml. of methyl alcohol on the tissue in the funnel, let stand a few minutes to ensure that the tissue is thoroughly moistened; then reattach to the suction to draw off the wash solvent. Repeat the washing once more in the same manner, then discard the tissue.

If possible, the hearts should be obtained on the day the animals are slaughtered, but it is not necessary to grind and extract them on the same day. A convenient practice is to wash and drain the hearts as soon as received, store them in the refrigerator overnight, and complete the cutting and grinding on the following morning.

Drying.—Best results are obtained when tissue from not more than 5 hearts is dried on about 8 square feet surface; time required, about 4 hours.

Separation of Crude Fraction Containing Cardiolipin.—To the methyl alcoholic extracts, add 20-per-cent $BaCl_2$ until no further precipitate forms; the amount required is about 10 ml. to 2 liters of extract. Place in the cold room (3°–6°C.) overnight or longer with occasional shaking to facilitate flocculation. Decant the supernatant through a large filter paper and collect the bulk of the precipitate by centrifugation. Wash the precipitate twice with 95-per-cent methyl alcohol (U.S.P.).

The barium precipitates from the two extracts may be pooled and worked up together.

The filtrate from Extract 1, after removing the barium precipitate, is used as a source of lecithin (see p. 394). Filtrates from later extracts contain some lecithin but hardly enough to repay the labor of isolation.

Conversion of Crude Barium Salts to Sodium Salts.—The volumes of solvents and reagents mentioned refer to the approximate quantities required for the material obtained from 5 hearts by two extractions.

To the packed barium precipitate in the centrifuge cups add about four times its volume of ether and for each 100 ml. of ether add 50 ml. of 5-per-cent Na_2SO_4 solution. Mix thoroughly, breaking up lumps with vigorous stirring. If necessary add more ether and Na_2SO_4 until the mixture separates in two layers. Centrifuge and transfer the liquid layers to a separatory funnel. Combine the precipitates in a single centrifuge cup and wash by mixing thoroughly with 100 ml. of ether, 15 ml. of methyl alcohol and 50 ml. of 5-per-cent Na_2SO_4. Centrifuge, discard the precipitate, and add the washing to the main portion of the solution. Separate and shake the ether layer vigorously for five minutes with 5-per-cent Na_2SO_4. Repeat, re-extracting the aqueous layers once with ether before discarding them, and finally dry the ethereal solution overnight on anhydrous Na_2SO_4. If emulsions form when the ethereal solution is shaken with Na_2SO_4, add just enough alcohol to break the emulsion.

The ethereal solution of crude sodium salts is contaminated with insoluble materials, $BaSO_4$ and organic impurities, which form persistent colloidal suspensions and interfere with filtration. For this reason the dried ethereal solution should be separated from the Na_2SO_4 by decantation: wash the Na_2SO_4 several times by decantation and finally pour it on a filter paper to separate the last wash ether. The ethereal solution is usually cloudy but this does not matter as the colloidal impurities are removed in the next step.

Concentrate the dried ether solution of sodium salts by distillation and finally to dryness *in vacuo*. To the residue in the distilling flask add about 75 ml. of acetone, warm in a bath at about 50°C., shake vigorously; separate by decantation and repeat the acetone treatment once more. Cool the acetone solution to room temperature and add any precipitate to the main acetone-insoluble fraction. Dissolve the acetone-insoluble fraction in 100–150 ml. of ether and pour the solution into 1 liter of methyl alcohol with rapid mixing. The precipitate should separate as a finely flocculent solid; if it is sticky, redissolve it in ether and again pour into methyl alcohol. Wash the final precipitate twice with warm methyl alcohol, then discard it.

Concentrate the ether-methyl alcoholic solution *in vacuo* to about 800 ml. to remove ether. The methyl-alcoholic solution so prepared is Fraction Me-1.

Purification of Cardiolipin.—A. *First Stage. Purification over the Barium Salt.* To the methyl-alcoholic solution of Fraction Me-1 add 2 per cent of its volume of saturated NaCl. Chill, with frequent vigorous shaking, until a clear supernatant is obtained, preferably overnight. Separate by decantation or centrifugation, without attempting to wash the NaCl precipitate. (For treatment of the NaCl precipitate, Fraction Me-2, see below under secondary fractions.) To the supernatant add $BaCl_2$ until no further precipitate forms. Chill with occasional shaking until the precipitate settles readily leaving the supernatant clear. Centrifuge, wash the precipitated barium salt once with methyl alcohol, and once with acetone, and dissolve it in a minimum amount of ether. At this point, the typical appearance of a salt containing a large proportion of cardiolipin is a stiff gel. Do not attempt to dissolve the gel by further dilution with ether; the amount of ether should be just sufficient to give a fairly clear and homogeneous gel on thorough mixing. To this gel add an equal volume of acetone in small portions with stirring. This precipitates the barium salt of cardiolipin while impurities remain in the ether-acetone supernatant.

When the salt is first taken up in ether, a suspension is sometimes obtained rather than a gel, due to the presence of excess acetone from the washing. Such a suspension may be precipitated with acetone as described above and the precipitate will yield a typical gel on taking up in ether for the second precipitation.

Centrifuge and repeat the ether-acetone precipitation until the supernatant is colorless or nearly so. Finally dissolve the precipitate in ether saturated with water, using about half as much wet ether as was used for the previous precipitation from the anhydrous solvent. To the clear ether solution add 2 volumes of acetone with rapid mixing. Centrifuge.

The precipitate, Fraction Ba-1, now consists of nearly pure barium cardiolipin. It is next converted to the sodium salt by the following procedure, which is of general application and is used also for conversion of the finally purified cadmium salt to the sodium salt (see p. 394).

Conversion to Sodium Salt. Dissolve the barium salt in wet ether containing 10 per cent of its volume of alcohol. The importance of adding alcohol lies in the fact that unless alcohol is present the reaction does not go to completion. The alcohol also prevents emulsification.

Shake the solution in a separatory funnel for five minutes with one-third of its volume of one-half saturated sodium chloride. Separate, add 10 ml. alcohol for each 100 ml. ether, and shake with NaCl as before. Repeat a third time, re-extracting the aqueous layers once with ether before discarding them. Then wash the ether layer twice by shaking with NaCl alone, in order to remove most of the alcohol. Dry the ethereal solution overnight on a little anhydrous Na_2SO_4, then filter and concentrate it by distillation to a small volume.

Solution of Sodium Salt. Pour the distillation residue into about 10 volumes of absolute alcohol with rapid mixing. To remove ether, concentrate the ether-alcohol solution *in vacuo* with the bath at about 45°C. until the distillate amounts to about 20 per cent of the original volume. At this point there is usually a small flocculent precipitate which may be centrifuged off, washed with alcohol, and discarded. However, if there should be more than a trace of this precipitate, it may mean that insufficient alcohol is present to hold all the sodium cardiolipin in solution; in such a case the precipitate should be redissolved in ether and again poured into 10 volumes of absolute alcohol. The solubility of sodium cardiolipin in absolute alcohol is approximately 10 mg. per ml. at 3°C., and it is therefore important to be sure that the volume of alcohol used is sufficient to yield a final concentration lower than this. Table 21 presents the yields of Fraction Ba-1 obtained in a typical preparation. From these data the amount of material to be expected, and therefore the volume of alcohol required, may be estimated.

Usually Fraction Ba-1 contains traces of insoluble impurities which separate slowly, and the absolute alcohol solution should be allowed to stand at from 3° to 6°C. until clear. The solution should be colorless or nearly so.

TABLE 21

Approximate yields of Fraction Ba-1 per kilogram fresh tissue

FROM	Me-1	Ba-2	Me-2	TOTAL
Ba-1	0.6 gm.	0.1–0.2 gm.	0.2–0.3 gm.	0.9–1.1 gm.

Secondary Fractions. A second crop of cardiolipin may be obtained by repurifying two of the secondary fractions from the first purification described above.

1. Ether-acetone supernatants from purification of the barium salt (Fraction Ba-2).

Concentrate the ether-acetone solution *in vacuo* to about one-half the original volume. Chill the mixture in ice, centrifuge, discard the acetone supernatant, and dissolve the precipitate in wet ether. Add 10 ml. alcohol for each 100 ml. of ether and convert to the Na salt by treatment with NaCl according to the procedure described for the purified salts (p. 391). Pour the concentrated ether solution into about 8 volumes of methyl alcohol; if a precipitate forms, wash it thoroughly with warm methyl alcohol and discard it. Remove ether by vacuum distillation and prepare the barium salt from the methyl-alcoholic solution exactly as was done with the original Fraction Me-1, adding any NaCl-precipitated material to Fraction Me-2. Purify the barium salt by precipitation from ether with acetone, convert it to the sodium salt, and dissolve the latter in absolute alcohol.

2. NaCl-precipitated material (Fraction Me-2).

Dissolve the precipitate in methyl alcohol; if a portion fails to dissolve readily, dissolve it in ether and pour the ethereal solution into about 8 volumes of methyl alcohol. To the methyl-alcoholic solution in a distilling flask, add about 1/10 its volume of HCl (1:10) and concentrate rapidly *in vacuo* until most of the methyl alcohol is removed. Dilute the residue with about an equal volume of water and cool the mixture in ice for a few minutes. At this point it will be found that most of the material has separated as a sticky mass adhering to the glass. Pour the supernatant into a separatory funnel and extract it with petroleum ether; dissolve the residue in the flask in petroleum ether and add the solution to the mixture in the separatory funnel. Discard the aqueous layer. Evaporate the petroleum ether solution to dryness *in vacuo*, add about 10 ml. of absolute alcohol for each gram of material, and again evaporate to dryness. Repeat with another addition of absolute alcohol. To the dry residue, add 10 to 15 ml. of acetone per gram and warm in a water bath at about 50°C., with frequent shaking, until the residue melts under the solvent; then cool the mixture in ice, decant the acetone solution, and re-extract the residue once more with warm acetone. Finally, dissolve the residue in a minimum amount of ether and mix rapidly with 7 or 8 volumes of acetone. Chill, centrifuge, and discard the acetone-insoluble residue.

Concentrate the combined acetone solutions nearly to dryness *in vacuo*. In releasing the vacuum, admit air instead of carbon dioxide as usual, in order to avoid excessive contamination of the salt with $BaCO_3$. Dissolve the distillation residue in 95-per-cent alcohol and neutralize the solution to phenolphthalein with saturated aqueous $Ba(OH)_2$. Chill the mixture in ice to facilitate flocculation of the barium salt. Wash the salt first with methyl alcohol and then with acetone and purify it by precipitation from ether with acetone. Convert it to the sodium salt and dissolve the latter in absolute alcohol.

Since acid cardiolipin is unstable, the entire procedure from the original acidification through the neutralization with $Ba(OH)_2$ must be carried out rapidly. With the amount of material obtained from five hearts the operation can easily be completed in three hours. It must in any case be finished within a single day.

B. *Second Stage. Purification over the Cadmium Salt.* Pool the three lots of the sodium salt of Fraction Ba-1 and to the alcoholic solution add $CdCl_2$ until no further precipitate forms. Let stand in the cold overnight or longer with occasional vigorous shaking. Centrifuge and wash the precipitate once with absolute alcohol. Estimate the volume of the precipitated cadmium salt by measurement of a water-blank in a similar centrifuge cup. Add to the precipitate an equal volume of ether, mix thoroughly and centrifuge the thick suspension. Since much of the bulk of the precipitate is solvent, this procedure amounts to washing the salt with a mixture of equal parts alcohol and ether. In this mixture the cadmium salt of cardiolipin is only very slightly soluble. With a larger proportion of ether its solubility increases; hence it is important to adhere to the specifications on the volumes to be used. Treat the washed precipitate with sufficient ether to give a homogeneous gel, as in the case of the barium salt, and precipitate the gelatinous solution with an equal volume of acetone. Centrifuge and repeat. Dissolve the salt in wet ether, using $\frac{1}{2}$ to $\frac{1}{3}$ as much ether as was used for the previous precipitation from anhydrous ether. Remove any excess water as completely as possible with a fine capillary pipette. Add 3 volumes of acetone rapidly with vigorous mixing. The precipitate may tend to be slightly sticky but is easily converted to a fine hard solid if it is promptly triturated and mixed thoroughly with a spatula. Centrifuge and repeat the precipitation once more in the same manner. Convert the purified cadmium salt to the sodium salt and dissolve in absolute alcohol by the procedure described on page 391.

The yield is about 70 per cent of the amount of Fraction Ba-1. There should be no more than traces of alcohol-insoluble impurities to be removed at the final stage.

The procedure for the purification of cardiolipin is presented in diagrammatic form in Fig. 48.

Purification of Lecithin (36).—To the supernatant of Extract 1 after removal of the barium precipitate, add $CdCl_2$ until no further precipitate forms. Let stand in the cold overnight or longer, then collect the precipitate and wash several times with methyl alcohol and finally once with ethyl alcohol.

Suspend the precipitate in petroleum ether and extract the mixture with 80-per-cent alcohol.

It is convenient to proceed as follows: Estimate the approximate volume of the packed precipitate in each centrifuge cup and add one-fifth its volume of water. To this mixture add an approximately equal volume of petroleum ether; mix thoroughly and transfer the suspension to a separatory funnel, rinsing with a mixture of petroleum ether and 80-per-cent alcohol. The cadmium salt of lecithin is soluble in 80-per-cent alcohol saturated with petroleum ether but is not soluble in either solvent alone. For the lecithin precipitate from 5 kg. of beef-heart tissue use about 300 ml. of petroleum ether.

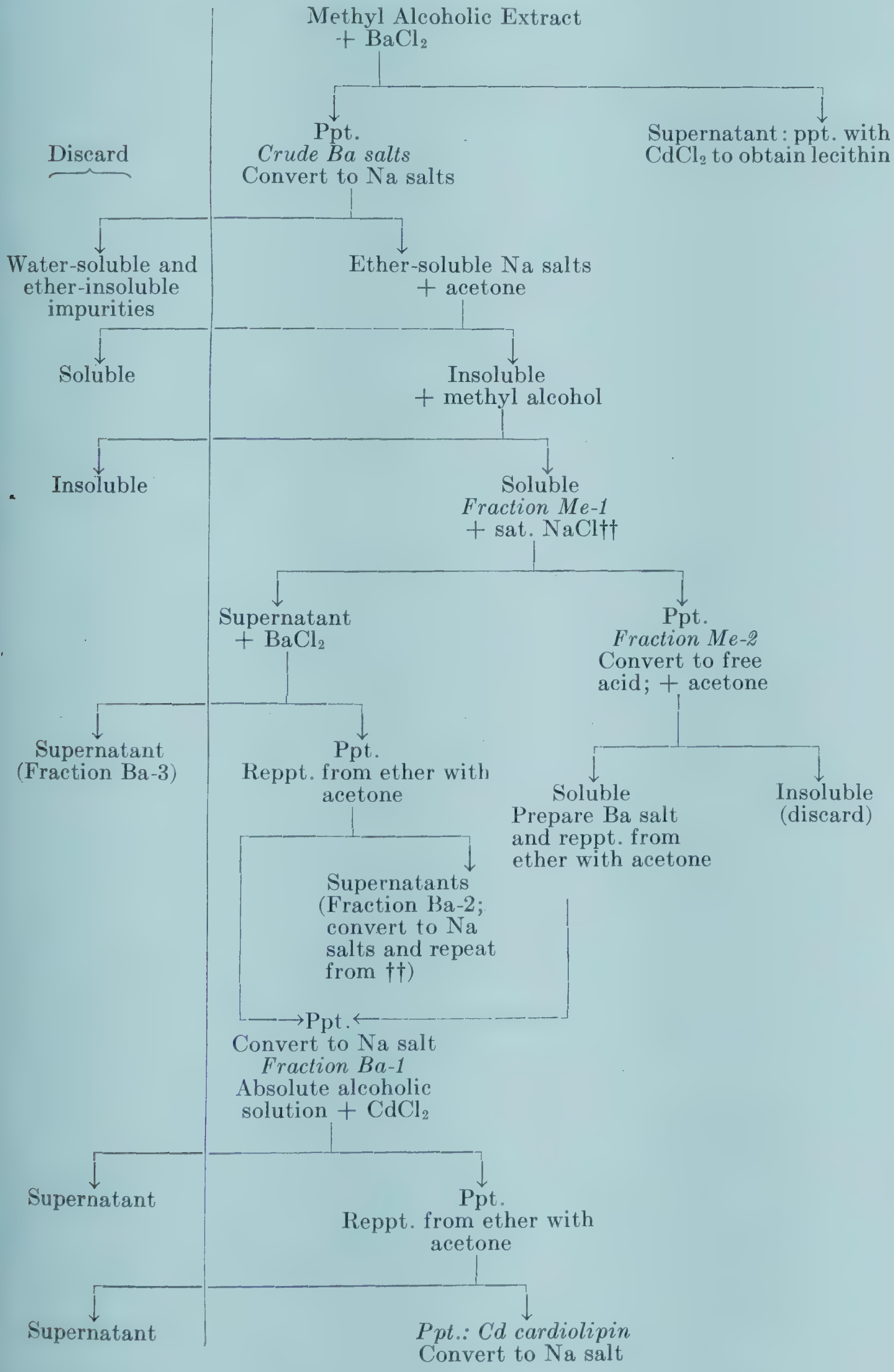
Continue the extraction with successive portions of 80-per-cent alcohol, using about one-fourth as much 80-per-cent alcohol as petroleum ether and adding petroleum ether frequently to keep the volume approximately constant. Shake each extraction vigorously for five minutes. Repeat until a small portion of the 80-per-cent alcoholic extract no longer gives a solid precipitate when the petroleum ether is removed by aeration and the solution cooled in ice. From 25 to 30 extractions may be required.

Toward the end of the extraction the test may give cloudy solutions or a sticky poorly settling precipitate; this is a negative test and such extracts should be discarded.

Combine all the 80-per-cent alcoholic extracts that have given satisfactory tests and concentrate *in vacuo* to about two-thirds of the original

FIGURE 48

PREPARATION OF CARDIOLIPIN


Methyl Alcoholic Extract
+ $BaCl_2$
Discard
Ppt.
Crude Ba salts
Convert to Na salts
Supernatant: ppt. with $CdCl_2$ to obtain lecithin
Water-soluble and ether-insoluble impurities
Ether-soluble Na salts
+ acetone
Soluble
Insoluble
+ methyl alcohol
Insoluble
Soluble
Fraction Me-1
+ sat. NaCl††
Supernatant
+ $BaCl_2$
Ppt.
Fraction Me-2
Convert to free acid; + acetone
Supernatant
(Fraction Ba-3)
Ppt.
Reppt. from ether with acetone
Soluble
Prepare Ba salt and reppt. from ether with acetone
Insoluble
(discard)
Supernatants
(Fraction Ba-2; convert to Na salts and repeat from ††)
Ppt.
Convert to Na salt
Fraction Ba-1
Absolute alcoholic solution + $CdCl_2$
Supernatant
Ppt.
Reppt. from ether with acetone
Supernatant
Ppt.: Cd cardiolipin
Convert to Na salt

volume. A heavy precipitate of the lecithin-cadmium salt separates as the petroleum ether is removed. A portion of the precipitate sometimes separates in a sticky mass adhering to the glass; such material should be dissolved in a mixture of petroleum ether and 80-per-cent alcohol and added to the cadmium precipitate in the second purification. Let the concentrated solution stand at 3°–6°C. overnight or longer, then collect the precipitate, suspend it in petroleum ether and repeat the extraction with 80-per-cent alcohol.

The above procedure is designed to give maximum purity rather than maximum yield, and results in considerable losses. The yield may be increased as follows: (a) After collecting the twice-purified cadmium salt, concentrate the second 80-per-cent alcohol supernatant further *in vacuo* to about one-half of its original volume and let stand in the cold for more of the cadmium salt to separate. (The supernatant from the *first* 80-per-cent alcohol purification should be discarded.) (b) With prolonged extraction there is apparently some dissociation of the lecithin-cadmium double salt, leaving free lecithin in the petroleum ether layer, from which it may be recovered as follows: Concentrate the petroleum ether layer nearly to dryness, dilute largely with alcohol and add $CdCl_2$ until no further precipitate forms. Collect the precipitate, suspend it in petroleum ether and extract with 80-per-cent alcohol as before. Combine the second crops of lecithin-cadmium salts, repurify by the petroleum ether 80-per-cent alcohol extraction and add to the first crop.

Collect the twice-purified cadmium salt by centrifugation, wash once with 95-per-cent alcohol, and dissolve in sufficient chloroform to give a clear solution. A satisfactorily purified preparation should yield a colorless solution at this point.

To the chloroform solution add a 20-per-cent solution of NH_3 in MeOH until the mixture is markedly alkaline to moist litmus.

Concentrated aqueous NH_4OH may be used if preferred. If this is done, however, it must be remembered that in the presence of water $Cd(OH)_2$ will redissolve in excess NH_4OH, hence care must be taken not to add too much ammonia.

If the precipitate of $Cd(OH)_2$ tends to be colloidal, add alcohol until it settles readily. Centrifuge; wash the precipitate once by mixing it with chloroform and then adding alcohol until the precipitate settles; this may require about equal parts of alcohol and chloroform.

To remove ammonia: Transfer the chloroform solution to a separatory funnel and shake vigorously with one-half saturated NaCl; separate; shake the chloroform solution again with NaCl, this time adding dilute HCl a few drops at a time, shaking vigorously between additions of acid, until the aqueous layer is neutral or barely acid when tested with fresh litmus. If emulsions form, add alcohol until the two layers separate readily. Separate and wash the chloroform solution once more by shaking vigorously for five minutes with one-half saturated NaCl. The final aqueous washing must be neutral.

Concentrate the washed chloroform solution to dryness *in vacuo*. Shake the residue in the distilling flask vigorously with a little acetone, decant, and repeat. After draining off the acetone as completely as possible, replace the flask in the water bath at about 45°C., evacuate for about ten minutes to remove residual acetone, then dissolve the lecithin in ether and transfer to a centrifuge cup. Avoid unnecessary dilution with ether; the amount of solvent used should be just sufficient to effect complete solution and quantitative transfer of the material. Let stand in the cold (3°–6°C.) overnight. Centrifuge for about one-half hour in a refrigerated centrifuge; wash the precipitate once with a little ice-cold ether, then discard it.

If a refrigerated centrifuge is not available, the ether solution may be chilled in a tall cylinder fitted with a siphon or in an Erlenmeyer flask supported at an angle to facilitate decantation. The insoluble material settles fairly well and most of the clear solution of lecithin can be siphoned or decanted off; the residue is then stirred up with ether and again allowed to settle for twenty-four hours. Filtration is not satisfactory.

Concentrate the ether solution by distillation, not quite to dryness, then add absolute alcohol and distill for a short time *in vacuo* to remove the residual ether. Filter the absolute alcohol solution if necessary to remove NaCl and dilute to the desired concentration. A 3-per-cent solution of lecithin should appear colorless when about 200 ml. of the solution is viewed through the depth of the column in a tall cylinder. If a yellow tint is noticeable, the lecithin should be repurified: dilute the solution with alcohol to a concentration of 1.5–2 per cent, precipitate with $CdCl_2$ and repeat the separation with petroleum ether and 80-per-cent alcohol. This repetition of the procedure is much more rapidly completed than the first purification, since fewer extractions with 80-per-cent alcohol are required when the lecithin is already fairly well purified. Only small amounts of colored impurities remain in the petroleum ether layer, which should be discarded. It is not necessary to repeat the step of dissolving in ether and chilling the solution: after removing chloroform and treating the residue with acetone, as described above, dissolve the dried lecithin directly in absolute alcohol.

Stock Solutions.—For methods of analysis, see chapter on "Other Methods of Quantitative Chemical Analysis and Precision Tests," p. 113. The solutions of cardiolipin and lecithin should be analyzed for phosphorus and nitrogen. Determination of the iodine number is also desirable.

The concentration of the stock solutions may be found by direct dry-weight determination or may be calculated from the phosphorus content. The latter method is more accurate. In calculating dry weight from P content assume, for cardiolipin, P = 4.18 per cent, and for lecithin, P = 3.95 per cent.

Nitrogen. Pure cardiolipin contains no nitrogen. If the amount found is greater than 0.1 per cent the material should be repurified over the cadmium salt.

The theoretical N:P ratio for lecithin is 1. In practice it has sometimes been found as high as 1.12 and lecithin containing this excess of nitrogen appears to be satisfactory for serologic use although a closer approach to the theoretical value is usually obtained. Lecithin purified as here described has regularly been found to contain less than 0.1 per cent amino nitrogen by the Van Slyke method; it is desirable however to analyze all preparations for amino nitrogen if possible as a further check on purity.

Iodine Number. Cardiolipin should have an iodine number not lower than 115; the usual range is from 118–122. Lower values indicate partial oxidation which lowers the serologic activity. Determination of the iodine number of lecithin is of interest but less essential. Values found for beef-heart lecithin have ranged from 75–85.

Preparation of Extract of Beef Heart

The antigen is a cholesterolized alcoholic extract of dried ether-extracted beef heart. It is important to eliminate anticomplementary and nonspecific activities that are due in large part to the presence of cholesterol and cephalin. First reduce the moisture content of the tissue to a minimum and then extract exhaustively with dry ether; otherwise there will be loss of specific activity.

Remove fat, blood vessels, pericardium, and endocardium from several fresh, normal, unincised beef hearts. Cut the tissue into small pieces and pass through a meat chopper. Spread in thin layers on a glass or stone surface and dry by a current of air from an electric fan. After the upper surface is dry, turn and continue drying until the brittle sheets can be pulverized in a corn mill or mortar. Dry the powdered tissue more completely over calcium chloride in a vacuum desiccator or by spreading in pans in a 37°C. incubator for three or four days. Extract the dried tissue exhaustively with anhydrous ether in a cold room. From six to nine extractions, for 2-day periods, in the proportion of 1000 grams of tissue to 2–3 liters of ether are required. Shake the ether-tissue mixtures mechanically for ten minutes daily. Remove the ether by filtration through soft paper. Place the residue in a vacuum desiccator in a warm bath (40°C.) until the odor of ether is no longer discernible. Mix the tissue with absolute alcohol in the proportion of 50 grams to 500 ml. and boil for one hour under a reflux condenser. Transfer the extracts and tissue to a container large enough to hold the entire batch. Mix thoroughly, allow to stand overnight, and then filter at room temperature.

TEST FOR EVIDENCE OF TUBERCULOSIS (5, 6, 8, 10, 27, 37, 38)

The antigen is prepared from a virulent strain of the tubercle bacillus of human origin and is a distilled-water extract of dried acetone-extracted microorganisms.

Cultures

Use glycerol-broth (*F8*) cultures of the tubercle bacillus grown to the point of optimum growth, that is, until the pellicle just covers the surface of the medium, which requires from three to four weeks. Sterilize the broth cultures by heating in an Arnold sterilizer for twenty minutes.

To separate the bacillary residue, filter the sterilized culture through soft filter paper. Then transfer it to a Büchner funnel and wash rapidly with large quantities of distilled water, using suction. Continue the suction until the microorganisms are dry and then desiccate over calcium chloride for two or three days. Store the dried bacilli in glass-stoppered bottles sealed with paraffin.

Distilled-Water Extract

Extract the washed and dried tubercle bacilli at room temperature for twenty-four hours with acetone, in the proportion of 100 ml. of dry C.P. acetone to each gram of microorganisms. Stir the mixture occasionally. Remove the acetone by filtration through hard filter paper and dry the microorganisms *in vacuo* over calcium chloride. Add distilled water in the proportion of 100 ml. to each gram of bacilli (weighed before treatment with acetone) and extract by boiling for one hour under a reflux condenser. Free the extract of sediment by centrifugation or filtration through sterile soft paper; sterilize the filtrate by heating for thirty minutes on three successive days at 56°C. If these antigens are stored in the refrigerator for several days, they may prove anticomplementary unless heated for one-half hour at 56°C. before use. They are always heated after use, for one-half hour on three successive days at 56°C., to maintain their sterility.

TEST FOR EVIDENCE OF GONOCOCCUS INFECTION (39)

A filtered extract of gonococcus cells disintegrated by alternate freezing and thawing is used as antigen. This extract is concentrated and washed by ultrafiltration.

The representative strains selected for antigen preparation are maintained on rabbit-blood agar (*F43A*). Transfers are made at intervals of three to four days.

Distilled-Water Extract Antigen

From the rabbit-blood-agar culture inoculate glucose ascitic-fluid-agar slants (*F43B*). Incubate for forty-eight hours at from 33° to 35°C. in the presence of 5- to 10-per-cent carbon dioxide. (See "General Bacteriologic Technic," p. 8.) Make slide preparations from each culture, stain by Gram's method (p. 35) and examine. Discard any contaminated or doubtful cultures. Pipette 5 ml. of physiologic salt solution into each tube; loosen the growth. Inoculate glucose-ascitic-fluid agar in pint Blake bottles (65 ml. in 1-pint bottle); use one tube of suspension for each bottle. Incubate for from twenty-two to forty-eight hours at approximately 33°C. Examine the cultures. Add about 7–8 ml. of 0.85-per-cent salt solution to each Blake bottle; loosen the growth by gentle agitation. Inoculate a blood-agar plate and make a slide preparation from each bottle. All cultures in which autolysis is marked or in which contamination is noted are discarded. Centrifuge the material from each bottle separately. Discard the supernatant fluid. Resuspend the bacterial sediment in about 8 ml. of sterile distilled water, dispense in 30-ml. bottles containing about 20 ml. of distilled water, and place in the freezing unit of an electric refrigerator.

Alternately freeze and thaw the preparations for six or seven days. Centrifuge those extracts found to be free from contamination. Pool the supernatant fluid, add a 1:10,000 dilution of phenyl mercuric acetate for a final concentration of approximately 1:200,000 and store in the cold. Resuspend the bacterial sediment in sterile distilled water, transfer to 30-ml. bottles containing a piece of sterile, washed glass wool, and again place in a freezing unit. Repeat the process until the centrifuged supernatant fluid, adjusted to isotonicity, produces no appreciable clouding with antigonococcus rabbit serum of high precipitative titer.

Filter the pooled supernatants through asbestos wool to clarify, then through a Mandler filter (see p. 806). Inoculate media (*F3B*) for tests of sterility (see p. 828). By filtration through a 4.5-per-cent nitrocellulose membrane (Parlodion) (see p. 815) concentrate the extract, wash with a volume of distilled water equal to that of the original extract and again concentrate. The volume of the residue should be approximately $\frac{1}{50}$ of that of the crude extract. Test the residue for sterility and remove a 1.0-ml. sample for testing for complement-fixing activity.

If the residue is sterile and if the titer is sufficiently high in complement-fixation tests, add phenyl mercuric acetate for a final concentration of 1:200,000 and 8.5-per-cent salt solution to isotonicity. Dispense in small bottles. Use aseptic technic in taking daily samples.

STANDARDIZATION OF ANTIGENS

CARDIOLIPIN-LECITHIN-CHOLESTEROL ANTIGEN

The experimental studies upon which the standardization of the cardiolipin antigen is based have been published separately (22, 22a). The standardization of the antigen for routine tests consists merely of mixing the given proportions of known solutions of the three components. As a check on the accuracy of the preparation of such mixtures, comparative tests of a few partially reacting syphilitic sera are made whenever a new mixture is prepared.

Three different lots of cardiolipin and two of lecithin have been used thus far in the preparation of antigen for use in complement-fixation tests and have given solutions of uniform reactivity.

The antigen at present in use, number 72, contains cardiolipin 0.0175 per cent, lecithin 0.0875 per cent, and cholesterol, 0.3 per cent. For convenience in preparing the antigen, a mixture of cardiolipin and lecithin of double this concentration, namely, cardiolipin 0.035 per cent, lecithin 0.175 per cent, is prepared. As needed, equal parts of this solution and of a 0.6-per-cent solution of cholesterol in absolute alcohol are mixed to make the antigen.

To prepare a mixture containing double the amounts of cardiolipin and lecithin that are present in the antigen, namely 0.035-per-cent cardiolipin and 0.175-per-cent lecithin, compute the amounts of the stock solutions of cardiolipin and lecithin that would be required as follows:

mg. per ml. in stock solution:1 ml.::mg. required to make the desired amount of the solution of double strength:x ml.

Place the amounts of the stock solutions of cardiolipin and lecithin, so determined, in a volumetric flask and make up to volume with absolute or 95-per-cent alcohol.

For example, if stock solutions of cardiolipin and lecithin contain respectively 9.54 mg. per ml. and 30 mg. per ml., the amounts of each required to prepare 1 liter of a mixture containing 0.035-per-cent cardiolipin (350 mg. per liter) and 0.175-per-cent lecithin (1750 mg. per liter) are found as indicated:

9.54 mg.:1 ml.:: 350 mg.:x ml.

$9.54\,x = 350$

$x = 36.69$ ml. of the given stock solution of cardiolipin

30 mg.:1 ml.::1750:x

$30\,x = 1750$

$x = 58.33$ ml. of the given stock solution of lecithin

To prepare the antigen mix the solution containing 0.035-per-cent cardiolipin and 0.175-per-cent lecithin with an equal part of a 0.6 per cent solution of cholesterol in absolute alcohol.

Stock solutions of cardiolipin and lecithin as well as the mixture of the two are stored in the refrigerator and should be allowed to come to room temperature—about 22°C.—before use. The alcoholic solution of cholesterol and the antigen, after cholesterolization, are stored at room temperature. All solutions are kept in amber glass bottles with screw tops lined with tin foil.

To dilute the antigen, place the required amount of salt solution in one beaker and pipette the antigen to the bottom of another; then add the salt solution to the antigen as rapidly as possible and mix thoroughly by pouring from one beaker to the other several times. The dilutions in salt solution shown in table 22 are used.

TABLE 22

Dilutions of cardiolipin antigen used in different types of test

FOR TESTS OF	WITH DILUTIONS OF COMPLEMENT CONTAINING			
	3 units	6 units	9 units	12 units
Undiluted serum	1:133	1:67		1:33
Undiluted cerebrospinal fluid	1:266	1:90		1:33
Endpoint titrations of				
Diluted serum	1:266, 1:133	1:90, 1:67	1:67, 1:45	1:45, 1:33
Diluted cerebrospinal fluid	1:400, 1:266	1:133, 1:90	1:67, 1:45	1:45, 1:22.5
Abbreviated technic of determining titer of				
Diluted serum		1:67	1:45	
Diluted cerebrospinal fluid		1:90	1:45	

OTHER ANTIGENS

Other antigens are titrated before use for lytic and anticomplementary properties and carefully standardized to determine the maximally reactive dose.

Dilute the cholesterolized alcoholic extract of beef heart used in the complement-fixation test for syphilis as follows: Place the required amount of salt solution in one beaker and pipette the antigen to the bottom of another; then add the salt solution to the antigen as rapidly as possible and mix thoroughly by pouring from one beaker to the other several times.

On the day the tubercle antigen is to be used, make it isotonic by adding one part of 8.5-per-cent sodium chloride solution to nine parts of antigen. Dilute the tubercle and gonococcus antigens by adding to one part of the isotonic antigen the amount of physiologic salt solution necessary to make the required dilution.

Titration of Hemolytic Properties

Antigens are occasionally hemolytic in low dilutions. To detect this property, test aqueous antigens undiluted after they have been made isotonic, and alcoholic extracts in a 1:10 dilution, since no dilution more concentrated than this would be used in any case. Perform the titrations as illustrated in table 23.

TABLE 23

Titration of hemolytic properties of antigen

TUBE NO.	ANTIGEN*	COMPLEMENT (3 UNITS)	SHEEP CELLS (5 PER CENT)	SALT SOLUTION	PERIOD FOR HEMOLYSIS
	ml.	*ml.*	*ml.*	*ml.*	
1	0.4	0.1	0.1	0	Standard for the test, see p. 372–374
2	0.2	0.1	0.1	0.1	
3	0.1	0.1	0.1	0.2	
4	None	0.1	0.1	0.3	
5	None	None	0.1	0.4	

* Lowest dilution that can be tested.

Centrifuge the tubes to deposit the cells, and record the degree of hemolysis in each.

If there is no hemolysis in the control tubes 4 and 5, but marked hemolysis in tubes 1 to 3 containing antigen, the extract is unsatisfactory for use in quantitative tests.

Titration of Anticomplementary Properties

Antigens, if properly prepared, will have no appreciable anticomplementary properties. Perform tests for these properties as illustrated in table 24.

Record the degree of hemolysis in each tube of the test. The control of sensitized cells (tube 5) should show no hemolysis; comparison of the reaction in the tubes without antigen, 3 and 4, with that in the tubes with antigen, 1 and 2, affords a measure of anticomplementary activity. Antigen that has more than slight anticomplementary action is unsatisfactory for use in the quantitative test.

TABLE 24

Titration of anticomplementary properties of antigen

TUBE NO.	ANTIGEN*	COMPLEMENT UNITS		SALT SOLUTION	FIXATION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
		1	2				
1	0.1	0.1	None	0.1	Standard for the test, see p. 372–374	0.2	Standard for the test, see p. 372–374
2	0.1	None	0.1	0.1		0.2	
3	None	0.1	None	0.2		0.2	
4	None	None	0.1	0.2		0.2	
5	None	None	None	0.3		0.2	

* Lowest dilution that can be tested.

Determination of Maximally Reactive Dose

The standardization of antigen consists in determining the dose or doses of antigen required to give the maximum reaction obtainable with such amounts of antibody or reagin as will be titrated with each of the amounts of complement used. In the case of antigens that give prozone reactions, that is, when an excess of antigen inhibits the reaction, it has been found necessary to use a different dose of antigen with each quantity of complement. For example, the dose of antigen that gives maximum reaction with an amount of reagin that reacts with a large dose of complement is so great as to inhibit the reaction of a smaller quantity of reagin, such as would react only with a smaller quantity of complement. The reverse is also true, *i.e.*, an amount of antigen small enough to give the maximum reaction with the lesser amount of reagin is insufficient to bring out the maximum reaction of the larger amount of reagin. See tabulation of antigen dilutions used, table 22. In the case of antigen that does not inhibit the reaction when present in excess, such as that described for use in the complement-fixation test for evidence of tuberculosis, the same dose can be used with all amounts of complement. Whenever it is an economy to do so, however, a smaller dose than is required for use with the larger amounts of complement may be used in tests with the smaller amounts of complement.

Cholesterolized Tissue Extract Antigens.—Standardization of tissue extract antigens prepared as described above, has been simplified materially (40, 41). It was found that standardization may be based upon the optimum ratio, cholesterol:extract, that exhibits maximum antigenic activity. When the concentration of antigen in a given alcoholic extract was lowered by diluting it in absolute alcohol, the amount of cholesterol required for optimum sensitization was proportionally reduced. For example, an extract that undiluted was optimally sensitized with 0.6-per-cent cholesterol, after dilution to 1:2 with alcohol was optimally sensitized with 0.3-per-cent cholesterol. The maximum reactions obtainable with both portions of the extract were the same. The saline dilutions however that provided the maximum reaction were twice as great in the case of the undiluted extract sensitized with 0.6-per-cent cholesterol as with the antigen diluted to 1:2 with alcohol and sensitized with 0.3-per-cent cholesterol. Similarly, other extracts, like the different concentrations of a given extract, when optimally sensitized, yielded similar titers in tests of syphilitic sera. Hence it is possible to maintain a constant standard activity of antigen in the complement-fixation test for syphilis.

The direct proportionality found between the optimum cholesterol concentration and the saline dilution of antigen that elicits a given maximum reaction simplifies standardization. The optimum ratio, cholesterol:extract, must be established for each preparation. If all antigens are then so diluted in alcohol as to be in optimum ratio with a constant amount of cholesterol, the same dilutions of antigen in saline will be optimum for use in tests. For example, if antigens are all diluted in alcohol so as to be in optimum ratio with 0.4-per-cent cholesterol, dilutions in saline to 1:100, 1:50, and 1:25 will be optimum for use respectively with 3, 6, and 12 units of complement. A brief series of comparative tests to check the suitability of these pre-established dilutions completes the standardization of the antigen.

To determine the optimum ratio, cholesterol:extract, antigen must be tested in concentrations such that the optimum amount of cholesterol will fall within the range of quantities that are soluble in the extract. Experience has shown that the concentration of reactive substances in different extracts, prepared as herein described, is quite similar. The optimum ratio, cholesterol:extract, can usually be determined therefore by testing several portions of a single dilution of extract in alcohol—for example, one part of extract diluted with 2 parts of absolute alcohol, each containing a different amount of cholesterol ranging from 0.2 to 0.5 gram per 100 ml. as shown in table 26. Crystallized cholesterol[6] may be added, in the given amounts, to portions of the antigen diluted to 1:3 in alcohol and allowed to dissolve at room temperature, or, 15-ml. portions of solutions of corresponding composition may be made by mixing alcoholic extract of tissue, an 0.8-per-cent alcoholic solution of cholesterol and absolute alcohol in the amounts given in table 25.

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[6] The cholesterol furnished by some manufacturers has been found less satisfactory than that procured from other firms. Pfanstiehl cholesterol, C.P., is used at the present time. Thus far, one lot has been encountered which was unsuitable for use with the cardiolipin antigen. All mixtures prepared with it yielded unstable suspensions in saline. After purification, however, by recrystallization from alcoholic solution, this cholesterol appeared quite as satisfactory as other samples.

For convenience in determining the optimum ratio, cholesterol:tissue extract, the relationships between this ratio and the corresponding optimally reactive saline dilutions of antigens, as deduced from experimental studies, are given in table 26. The dilutions that must be made of the original extract in alcohol in order to bring it into optimum adjustment with 0.4-per-cent cholesterol when the optimum ratio, cholesterol:extract

TABLE 25

Preparation of tissue-extract antigen solutions for use in standardization

SOLUTION DESIRED	SOLUTIONS TO BE MIXED TO PREPARE 15-ML. PORTIONS			
Antigen diluted to 1:3 with absolute alcohol and sensitized with cholesterol	Alcoholic extract of tissue	0.8-per-cent solution of cholesterol in absolute alcohol		Absolute alcohol
		ml.	cholesterol equivalent	
%	*ml.*		*mg.*	*ml.*
0.2	5	3.75	30.0	6.25
0.25	5	4.69	37.5	5.31
0.3	5	5.62	45.0	4.38
0.35	5	6.56	52.5	3.44
0.4	5	7.50	60.0	2.50
0.45	5	8.44	67.5	1.56
0.5	5	9.37	75.0	0.63

TABLE 26

Data utilized in making the optimum adjustment, cholesterol:tissue extract

ALCOHOLIC EXTRACT OF BEEF-HEART TISSUE DILUTED 1:3 IN ABSOLUTE ALCOHOL (33.3% EXTRACT), SENSITIZED WITH CHOLESTEROL	RATIO CHOLESTEROL: EXTRACT IN SOLUTIONS OF COLUMN 1* (% CHOL.: % EXT.:: 1:X)	SALINE DILUTIONS OF ANTIGEN OPTIMUM FOR USE WITH DIFFERENT AMOUNTS OF COMPLEMENT WHENEVER THE GIVEN AMOUNTS OF CHOLESTEROL, COLUMN 1, ARE IN OPTIMUM RATIO WITH EXTRACT				DILUTION OF ORIGINAL EXTRACT IN ABSOLUTE ALCOHOL THAT MUST BE MADE TO BRING ANTIGEN INTO OPTIMUM RATIO WITH 0.4% CHOLESTEROL, WHEN THE OPTIMUM RATIO CHOLESTEROL: EXTRACT IS FOUND TO CORRESPOND TO ONE OF THE VALUES GIVEN IN COLUMN 2
		Units of complement				
		3	6	9	12	
%						
0.2	1:167	1:50	1:25	1:16.7	1:12.5	1:1.5
0.25	1:133	1:62.5	1:31.3	1:20.8	1:15.6	1:1.88
0.3	1:111	1:75	1:37.5	1:25	1:18.8	1:2.25
0.35	1:95.2	1:87.5	1:43.8	1:29.2	1:21.9	1:2.62
0.4	1:83.3	1:100	1:50	1:33	1:25	1:3.00
0.45	1:74.1	1:113	1:56.5	1:37.7	1:28.3	1:3.38
0.5	1:66.7	1:125	1:62.5	1:41.7	1:31.3	1:3.75

* In experience with five different extracts the optimum ratio, cholesterol:extract, was 1:74.1 with one, 1:83.3 with two, and 1:111 with two.

is found to correspond with any one of the values given in column 2 are also included.

Test the several portions of cholesterolized extract as shown in table 27, which records the results of typical titrations. Include tests of the anticomplementary properties of the different dilutions of the antigen, using 0.1-ml. amounts in the presence of 1 and 2 units of complement; controls of serum without antigen with 1 and 2 units of complement; and controls of 1 and 2 units of complement alone. When the sensitized cells are added to the tests, include also a control of sensitized cells to which salt solution alone is added. The controls of a given reagent need be included only in the first and last sets of tests made.

Record the degree of hemolysis obtained in each tube of the test. The control of sensitized cells should show no hemolysis; comparison of the reaction in the complement control with that in the antigen controls affords a measure of the anticomplementary activity of the antigen.

Evaluate the reactions in the different tubes of the test in terms of the number of units of complement required for 50-per-cent hemolysis by reference to table 28. The table gives these values for the different degrees of hemolysis that may occur with each of the quantities of complement tested.

The values correspond with those recorded in the rows opposite 50-per-cent hemolysis in the ratio charts, tables 35–46, described under "Reporting of Results," and include also values for reactions occurring with 1 and 2 units of complement.

Plot the maximum number of units of complement required for 50-per-cent hemolysis by each amount of serum in the presence of antigen directly against the amount of serum tested and fit a straight line to the plotted points as shown in figure 49. Relative antigenic activity is measured in terms of the slope of the line denoting the linear relation between serum and complement. In the example given in table 27 and figure 49 it is evident that with extract diluted 1:3 in alcohol addition of cholesterol beyond 0.3 per cent does not significantly increase the reaction. With larger amounts, equal or slightly diminished serum reactions are observed. The optimum ratio, cholesterol:tissue extract, therefore, would be 1:111 as indicated in table 26.

When the optimum cholesterol:antigen ratio has been determined, dilute a portion of the antigen in absolute alcohol so as to bring it into optimum ratio with 0.4-per-cent cholesterol (in the example given, tissue extract would be diluted in alcohol to 1:2.25 before sensitization with 0.4-per-cent cholesterol).

Test this portion of extract, in comparison with a previously standardized antigen, with 25 to 50 syphilitic sera of varying degrees of reactivity. Also, test the antigen as fully as possible with sera from persons with

TABLE 27

Results of titrations to determine the optimum ratio, cholesterol:tissue extract

SYPHILITIC SERUM DILUTED IN NORMAL SERUM*		ANTIGEN† 1:3 IN ALCOHOL, 0.5% CHOLESTEROL; DILUTION IN SALT SOLUTION 0.1 ML.								MAXIMUM REACTION WITH GIVEN ANTIGEN (READ FROM TABLE 28)‡
Dilution tested 0.05 ml.	Equivalent amount of serum	1:250	1:125	1:83	1:62.5	1:62.5	1:41.7	1:41.7	1:31.3	
		Units of complement in 0.1 ml.								
		3		6		9		12		
1:25	0.0020			10	5	85	50	95	*80*	10.1
1:31	0.0016			20	*15*	90	80	100	100	7.8
1:41	0.0012	10	0	*50*	*50*	100	98			6.0
1:62.5	0.0008	15	*10*	95	98					4.5
1:125	0.0004	*60*	90	100	100					2.8
		ANTIGEN 1:3 IN ALCOHOL, 0.45% CHOLESTEROL; DILUTION IN SALT SOLUTION 0.1 ML.								
		1:226	1:113	1:75	1:56.5	1:56.5	1:37.7	1:37.7	1:28.3	
1:25	0.0020			5	0	90	30	100	*80*	10.1
1:31	0.0016			15	*10*	100	80	100	100	8.3
1:41	0.0012	15	5	55	*50*	100	100			6.0
1:62.5	0.0008	25	*15*	95	100					4.1
1:125	0.0004	60	95	100	100					2.8
		ANTIGEN 1:3 IN ALCOHOL, 0.4% CHOLESTEROL; DILUTION IN SALT SOLUTION 0.1 ML.								
		1:200	1:100	1:67	1:50	1:50	1:33	1:33	1:25	
1:25	0.0020			5	0	60	20	*70*	80	10.8
1:31	0.0016			15	10	85	*60*	95	100	8.5
1:41	0.0012	10	0	60	*45*	98	100			6.2
1:62.5	0.0008	15	*5*	90	100					5.0
1:125	0.0004	*70*	85	100	100					2.5
		ANTIGEN 1:3 IN ALCOHOL, 0.35% CHOLESTEROL; DILUTION IN SALT SOLUTION 0.1 ML.								
		1:175	1:87.5	1:60	1:43.8	1:43.8	1:29.2	1:29.2	1:21.9	
1:25	0.0020			0	0	45	15	*60*	70	11.5
1:31	0.0016			*5*	*5*	50	90	95	100	9.1
1:41	0.0012	5	0	25	*20*	95	98			7.4
1:62.5	0.0008	*10*	20	90	95					4.5
1:125	0.0004	*60*	85	100	100					2.8
		ANTIGEN 1:3 IN ALCOHOL, 0.3% CHOLESTEROL; DILUTION IN SALT SOLUTION, 0.1 ML.								
		1:150	1:75	1:50	1:37.5	1:37.5	1:25	1:25	1:18.8	
1:25	0.0020			5	0	25	40	60	*50*	12.0
1:31	0.0016			10	*5*	70	80	90	100	9.1
1:41	0.0012	5	0	*35*	60	95	100			6.7
1:62.5	0.0008	*5*	20	95	100					5.0
1:125	0.0004	*75*	90	100	100					2.4

TABLE 27—*Continued*

SYPHILITIC SERUM DILUTED IN NORMAL SERUM*		ANTIGEN 1:3 IN ALCOHOL, 0:25% CHOLESTEROL; DILUTION IN SALT SOLUTION, 0.1 ML.								MAXIMUM REACTION WITH GIVEN ANTIGEN (READ FROM TABLE 28)‡
Dilution tested 0.05 ml.	Equivalent amount of serum	1:125	1:62.5	1:40	1:31.3	1:31.3	1:20.8	1:20.8	1:15.6	
1:25	0.0020			5	0	40	*15*	90	90	11.0
1:31	0.0016			15	10	80	*50*	98	100	9.0
1:41	0.0012	5	0	*50*	60	98	100			6.0
1:62.5	0.0008	20	*15*	100	98					4.1
1:125	0.0004	*70*	95	100	100					2.5
		ANTIGEN 1:3 IN ALCOHOL, 0.2% CHOLESTEROL; DILUTION IN SALT SOLUTION, 0.1 ML.								
		1:100	1:50	1:33	1:25	1:25	1:16.7	1:16.7	1:12.5	
1:25	0.0020	15	10	100	*75*	100	100			4.9
1:31	0.0016	20	25	100	*90*	100	100			4.0
1:41	0.0012	*25*	55	100	98					3.7
1:62.5	0.0008	*45*	60	100	100					3.1
1:125	0.0004	*80*	95	100	100					2.3

* Strongly reacting syphilitic serum, or pools of such sera, are diluted in pooled nonreacting sera, each dilution being made separately from undiluted syphilitic serum.

When cerebrospinal fluid is titrated, dilutions to 1:4, 1:5, or 1:10 are made in salt solution. Different amounts between 0.02 and 0.2 are pipetted. The total volume in all tubes of the test, before fixation, is brought to 0.4 ml. with salt solution.

The range of reactivity of the syphilitic serum or cerebrospinal fluid with standard antigen determines the dilutions of serum and the dilution and amount of cerebrospinal fluid to be used. This is found by a titer-range test as described on p. 438–440.

† Fixation is for four hours at from 3° to 6°C.; after the addition of 0.2 ml. sensitized cells to each tube, fifteen minutes in the water bath at 37°C. are allowed for hemolysis.

‡ The percentages of hemolysis representing the maximum serum reactions are *italicized.*

infections other than syphilis. For this purpose it is convenient here to use the antigen in preliminary complement-fixation tests of all specimens examined for a period of a week or more.

The titers obtained in tests of syphilitic sera with the new antigen should agree, within the limits of the accuracy of the technic,[7] with those found

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[7] The relative discrepancy—$\frac{\text{difference}}{\text{mean}}$—between successive observations on the same specimen, when expressed in two significant figures, should not exceed 10 per cent more than half the time and should not exceed 29 per cent more than once in ten times. The relative discrepancies between duplicate observations on a given day should not exceed 10 per cent more than one time in ten.

The anticomplementary activity of the patient's serum alone without antigen as well as that of the diluent when dilutions are used in the test, is a disturbing factor in determining the titer, which at present is the ratio between the activity of the serum without antigen and that with antigen. In the absence of appreciable anticomplementary activity, as in the majority of sera of low titer, the ratio appears to be a conservative method of expressing the titer. But in the exceptional instances of relatively high degrees of specific activity in known cases of syphilis with marked anticomplementary activity the ratio may be misleading; it accounts for extreme discrepancies which are masked by statistical analysis of the deviation of the test because these are few in number. The total fixation may be a truer index in these extreme cases, especially when the presence of syphilis is known and since the sig-

TABLE 28

Units of complement required for 50-per-cent hemolysis as indicated by the degrees of hemolysis obtained in tests with different amounts of complement

REAGENTS PRESENT	UNITS OF COMPLEMENT	PER CENT HEMOLYSIS																	
		5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
Test for syphilis—incubation for four hours at from 3° to 6°C.; fifteen minutes for hemolysis																			
Complement alone or with antigen	1	1.61	1.45	1.33	1.27	1.20	1.15	1.12	1.07	1.04	1.0	0.97	0.93	0.91	0.87	0.84	0.80	0.76	0.69
	2	3.22	2.90	2.67	2.53	2.41	2.30	2.25	2.15	2.08	2.0	1.94	1.87	1.82	1.74	1.68	1.60	1.52	1.39
With serum or with serum and antigen	1	1.78	1.54	1.39	1.32	1.23	1.19	1.12	1.09	1.04	1.0	0.96	0.93	0.88	0.85	0.81	0.76	0.71	0.65
	2	3.51	3.06	2.81	2.63	2.49	2.37	2.26	2.16	2.07	2.0	1.92	1.84	1.76	1.69	1.60	1.52	1.42	1.29
	3	5.02	4.45	4.12	3.88	3.69	3.52	3.37	3.24	3.10	3.0	2.86	2.75	2.64	2.52	2.40	2.26	2.12	1.92
With serum and antigen	6	9.05	8.25	7.75	7.40	7.20	6.80	6.65	6.35	6.20	6.0	5.76	5.59	5.39	5.16	4.93	4.65	4.37	3.98
	9	12.2	11.5	11.0	10.6	10.2	10.0	9.7	9.5	9.2	9.0	8.8	8.5	8.2	7.9	7.6	7.3	6.8	6.2
	12	15.0	14.4	14.0	13.5	13.3	13.0	12.7	12.5	12.2	12.0	11.7	11.5	11.2	10.8	10.5	10.1	9.5	8.8
Test for tuberculosis—incubation for one and one-half hours at 37°C.; sixty minutes for hemolysis																			
Complement alone or with antigen	1	1.45	1.32	1.24	1.19	1.15	1.11	1.09	1.05	1.03	1.0	0.98	0.95	0.92	0.90	0.87	0.84	0.81	0.76
	2	2.9	2.64	2.48	2.38	2.30	2.22	2.17	2.10	2.05	2.0	1.95	1.91	1.84	1.80	1.74	1.68	1.61	1.52
With serum	1	1.89	1.59	1.43	1.33	1.25	1.19	1.15	1.09	1.04	1.0	0.96	0.92	0.88	0.84	0.79	0.75	0.70	0.63
	2	3.77	3.17	2.86	2.67	2.50	2.38	2.30	2.17	2.08	2.0	1.92	1.84	1.75	1.68	1.59	1.49	1.40	1.27
With serum and antigen	2	3.02	2.75	2.59	2.46	2.35	2.27	2.20	2.12	2.06	2.0	1.94	1.87	1.82	1.75	1.69	1.62	1.53	1.42
	3	4.36	4.01	3.80	3.62	3.50	3.38	3.28	3.18	3.09	3.0	2.91	2.82	2.73	2.63	2.53	2.43	2.30	2.14
	6	8.03	7.50	7.20	6.90	6.75	6.55	6.40	6.25	6.15	6.0	5.85	5.70	5.59	5.43	5.29	5.10	4.86	4.55
	9	11.2	10.7	10.3	10.1	9.9	9.6	9.4	9.3	9.1	9.0	8.8	8.6	8.4	8.2	8.0	7.8	7.5	7.1
	12	14.1	13.7	13.3	13.0	12.8	12.6	12.5	12.3	12.1	12.0	11.8	11.6	11.4	11.2	11.0	10.7	10.3	9.9

Test for gonococcus infection—incubation for one and one-half hours at 37°C.; fifteen minutes for hemolysis

Complement alone	1	1.43	1.30	1.24	1.19	1.15	1.12	1.08	1.06	1.03	1.0	0.98	0.96	0.94	0.91	0.88	0.85	0.82	0.77
	1.5	2.15	1.95	1.86	1.79	1.73	1.68	1.62	1.59	1.55	1.5	1.47	1.44	1.41	1.37	1.32	1.28	1.23	1.16
Complement with antigen	1	1.54	1.39	1.29	1.22	1.17	1.13	1.09	1.06	1.03	1.0	0.97	0.95	0.92	0.89	0.86	0.82	0.78	0.73
	1.5	2.31	2.09	1.94	1.83	1.76	1.70	1.64	1.59	1.55	1.5	1.46	1.43	1.38	1.34	1.29	1.23	1.17	1.10
Complement with serum	1	1.59	1.41	1.31	1.24	1.19	1.14	1.10	1.06	1.03	1.0	0.97	0.94	0.91	0.87	0.84	0.81	0.76	0.71
	1.5	2.39	2.12	1.97	1.86	1.79	1.71	1.65	1.59	1.55	1.5	1.46	1.41	1.37	1.31	1.26	1.22	1.14	1.07
Complement with serum and antigen	2	3.18	2.81	2.61	2.48	2.38	2.29	2.20	2.12	2.06	2.0	1.94	1.88	1.82	1.74	1.68	1.62	1.52	1.42
	3	4.57	4.23	3.90	3.77	3.57	3.45	3.31	3.20	3.09	3.0	2.92	2.83	2.77	2.67	2.55	2.45	2.32	2.15
	4	5.87	5.37	5.10	4.83	4.63	4.53	4.38	4.23	4.17	4.0	3.87	3.80	3.67	3.53	3.40	3.27	3.10	2.87
	6	8.20	7.73	7.35	7.07	6.83	6.67	6.50	6.31	6.20	6.0	5.83	5.72	5.57	5.35	5.13	4.93	4.63	4.37
	8	10.3	9.80	9.43	9.13	8.87	8.73	8.50	8.30	8.17	8.0	7.87	7.67	7.40	7.20	6.97	6.73	6.40	6.00
	12	14.3	13.9	13.3	13.1	12.8	12.7	12.5	12.3	12.1	12.0	11.9	11.6	11.4	11.2	10.9	10.6	10.2	9.67

with the standard antigen and there should be no evidence of increased reaction with sera of nonsyphilitic donors. Otherwise recheck the adjustment of cholesterol or, if necessary, prepare and standardize a new extract.

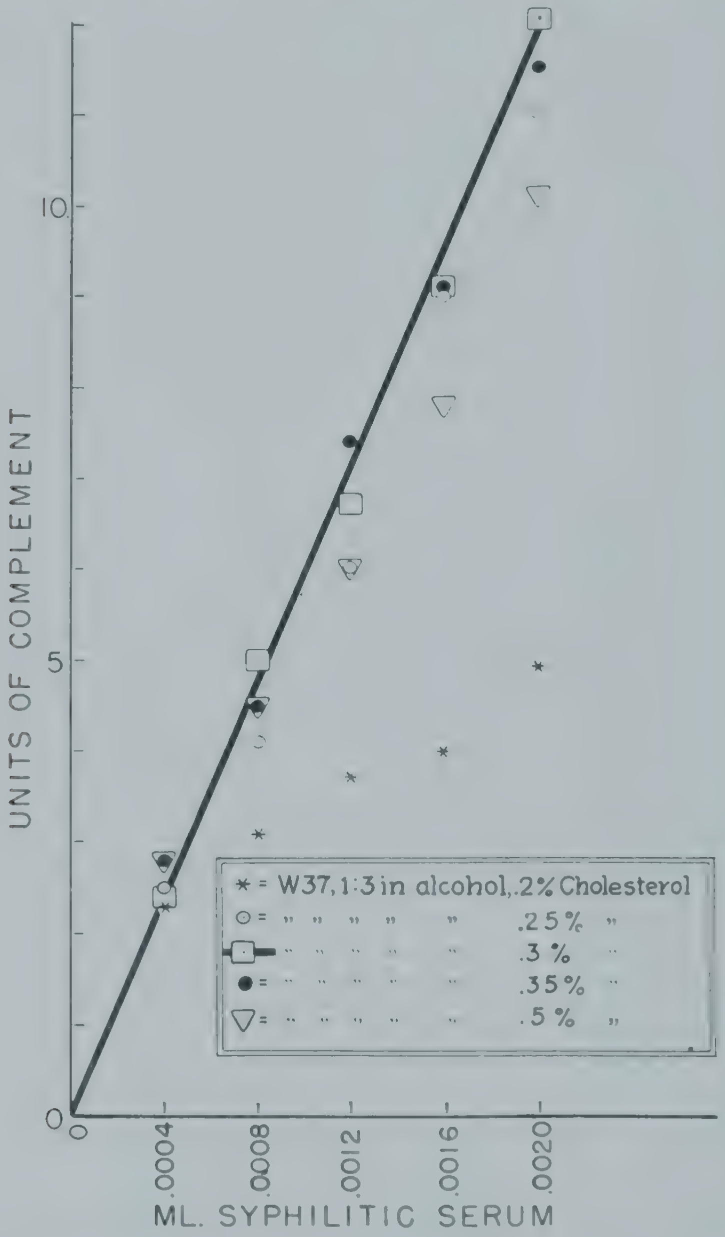

FIG. 49. DETERMINATION OF OPTIMUM RATIO CHOLESTEROL: TISSUE EXTRACT

Determine in the same manner as in tests of serum the optimum adjustment, cholesterol:tissue extract, for use in tests of cerebrospinal fluids. In experience thus far the same ratio cholesterol:extract has been optimum

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nificance of the anticomplementary activity in its relation to the specific activity is not known. In any event, the degrees of anticomplementary activity are essential controls. Extremes of anticomplementary activity occur in cases of syphilis without destroying the linear relationships of the specific reaction of complement fixation. Also, they occur more frequently in the later than in early stages of the disease for use with both. Somewhat higher saline dilutions of antigen than are used with serum however have been found optimum for use with cerebrospinal fluids. For example, antigen that is diluted 1:100, 1:50, and 1:25 for use in the test of serum with 3, 6, and 12 units of complement, respectively, is diluted to 1:200, 1:100, and 1:33 for use in tests of cerebrospinal fluid.

Tubercle and Gonococcus Antigens.—To standardize these antigens, titrate the respective immune sera as shown in table 29, using several

TABLE 29

Serum titration

STRONGLY REACTING PATIENT'S SERUM OR IMMUNE SERUM 0.05 ML.	SERUM WITHOUT ANTIGEN			TESTS WITH 0.1 ML. OF REQUIRED DILUTIONS OF ANTIGEN* UNITS OF COMPLEMENT IN 0.1 ML.†			
	1	2	3	3	6	9	12
Undiluted	x	x	x				
1st dilution‡	x	x	x	x	x	x	x
2d dilution‡				x	x	x	x
3d dilution‡				x	x	x	x
4th dilution‡				x	x	x	x
5th dilution‡				x	x	x	x

x = Tests made with each quantity of serum. Salt solution is added in amounts to bring the total volume in each tube to 0.3 ml.

Period of fixation = standard for the test. See p. 372–374.

After the addition of 0.2 ml. sensitized cells to each tube, the period of incubation at 37°C. that is standard for the test, see p. 372–374, is allowed for hemolysis.

When the titration is made with a standard antigen certain tubes may be omitted. For example:

WITH SERUM DILUTION	OMIT TUBES CONTAINING UNITS OF COMPLEMENT
1	3 and 6
2	3
4	12
5	9 and 12

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* Use the dilution that contains in 0.1 ml. the maximally reactive dose of a standard antigen. When this type of titration is made in standardizing a new antigen perform similar titrations with each of several different dilutions usually ranging, in the case of the tubercle antigens from 1:5 to 1:20 and of the gonococcus antigens from 1:5 to 1:40.

† Substitute in the titrations with gonococcus antigens, dilutions of complement containing 2, 3, 4, 6, 8, and 12 units. Add the reagents in the following order: serum, complement, antigen, salt solution.

‡ Serum is diluted according to its range of reactivity as found in a preliminary titer-range test, see p. 438. Each dilution is prepared from the original serum. The

TABLE 29—*Concluded*

first dilution should contain in 0.05 ml. about 4 to 6 times as much of the serum to be tested as the 5th dilution and the others are spaced to give suitable increments between the two extremes. One of the following dilution series is usually used but may need to be modified for use with particular sera or increased 10-fold as sera of higher titer are encountered.

1:1.25	1:1.67	1:2.5	1:4	1:5	1:8	1:10
1:1.67	1:2.5	1:3.1	1:5	1:6.25	1:10	1:12.5
1:2.5	1:3.33	1:4.1	1:6.67	1:8.33	1:13.3	1:16.7
1:3.33	1:5	1:6.25	1:10	1:12.5	1:20	1:25
1:5.0	1:10	1:12.5	1:20	1:25	1:40	1:50

For accurate results the use of a satisfactory range of serum dilutions is essential. The largest amount of serum tested should give some degree of partial hemolysis with 12 units of complement; the smallest amount, partial hemolysis with 3 units. Allowance for color of the test serum or other reagents is important at all times and particularly so in evaluating end points based on 5–10-per-cent hemolysis with 12 units of complement in these titrations. When normal or nonreacting serum is used as diluent of the immune serum, it should be so selected that the anticomplementary properties are minimal. Filtered pools of nonreacting human sera are so frequently anticomplementary that freshly collected, unfiltered, pools are used by preference. Serum diluent should always be tested with the antigen in use. See tables 32, 33, or 34; omit the tests with 6 and 12 units of complement but add a test with 2 units.

different dilutions of the new antigen in comparison with the maximally reactive dose of a previously standardized antigen. Include tests of the anticomplementary properties of the different dilutions of the antigens, using 0.1-ml. amounts in the presence of 1 and 2 units of complement and controls of 1 and 2 units of complement alone; and also a control of 0.2 ml. of sensitized cells to which salt solution alone is added.

Evaluate the reactions in the different tubes of the titrations in terms of the number of units of complement required for 50-per-cent hemolysis by reference to table 28. Plot the results obtained in each titration using the maximum number of units of complement required for 50-per-cent hemolysis in the presence of antigen and the different quantities of serum as ordinates against the corresponding quantities of serum as abscissae. Select as maximum that dose of antigen beyond which further increase fails to yield a significantly greater degree of reaction with the serum tested.

A new antigen is compared with a previously standardized antigen in parallel quantitative tests of appropriate sera of varying degrees of reactivity. These should include specimens from persons with different forms and in different stages of a given disease, or under treatment, and also from persons with other infections and from apparently healthy individuals. The titers of reacting sera with the new and the standard antigens should agree within the limits of accuracy of the technic (see footnote 7, p. 409) and the new antigen should show no increased tendency to react with sera from donors with miscellaneous types of infection.

DETERMINATION OF CONVERSION FACTORS

The conversion factors for use with the present antigens are given in table 30. New conversion factors need to be determined only if the effect of a new antigen upon the slope of the hemolytic curve differs from that of the standard antigen. Such an effect is indicated if plots of the reactions with the new antigen, based on the values given in table 28, do not show a linear relationship between serum and complement. Failure of the linear relationship in these graphs, however, may be due to other causes; for example, to the use of dilutions of the antigen other than those that are maximally reactive, or possibly to some property peculiar to the antigen under investigation. When it is necessary to determine new conversion factors for use with a new antigen, titrations with amounts of guinea-pig serum ranging from 0.04 to 0.001 ml. in a geometric progression based on 0.9 should be made under conditions requiring various amounts of complement for 50-per-cent hemolysis; namely, in the presence of appropriate quantities of reacting serum and of the dilutions of antigen that appear to be maximally reactive.

Prepare a 1:2.5 dilution of pooled complement in a French square bottle of about 100-ml. capacity by mixing 4 ml. of undiluted complement with 6 ml. of physiologic salt solution. Mix by tilting and gently rotating the bottle. Remove one-tenth of the total volume, that is, 1 ml., to the first of a series of test tubes and replace with 1 ml. of salt solution. Mix gently and remove 1 ml. to the second tube in the series and replace with 1 ml. of salt solution as before. Repeat the process until thirty-six dilutions are prepared. If a larger amount than 1 ml. of each of the complement dilutions is required, the amount of the original 1:2.5 dilution may be increased proportionally, as long as the progression of 0.9 is maintained in making the dilutions.

Pipette the reagents in the following amounts: complement, 0.1 ml. of the required dilutions (for this purpose, use the upper tenth of a 0.2-ml. pipette, a separate pipette for each dilution, and deliver to the bottom of each tube); reacting serum of known titer in appropriate dilutions, 0.05 ml.; antigen, 0.1 ml.; salt solution, 0.05 ml.[8] After fixation, add 0.2 ml. sensitized cells and complete the titrations in the usual way.

Plot the different percentages of hemolysis obtained against the amounts of complement tested. Fit a smooth curve by inspection to each titration. From those titrations in which the quantities of complement required for 50-per-cent hemolysis correspond approximately to the amounts repre-

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[8] In the complement-fixation test for syphilis somewhat less fixation occurs when reagents are mixed in this order; however the slope of the curve expressing the hemolytic reaction remains unchanged.

TABLE 30

The amount of complement required for a given degree of hemolysis relative to that for 50-per-cent hemolysis

REAGENTS PRESENT	RELATIVE VOLUME OF COMPLEMENT REQUIRED FOR 50% HEMOLYSIS	VALUE OF $1/n$ IN VON KROGH'S FORMULA $x = K\left(\frac{y}{1-y}\right)^{1/n}$	PER CENT HEMOLYSIS																	
			5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
			Conversion factors																	
Test for syphilis—incubation for four hours at from 3° to 6°C.; fifteen minutes for hemolysis																				
Complement alone or with antigen	1 to 2	0.165	0.62	0.69	0.75	0.79	0.83	0.87	0.89	0.93	0.96	1.0	1.03	1.07	1.10	1.15	1.19	1.25	1.32	1.44
With serum or with serum and antigen	1 to 3	0.196	0.56	0.65	0.72	0.76	0.81	0.84	0.89	0.92	0.96	1.0	1.04	1.08	1.13	1.18	1.24	1.32	1.40	1.54
With serum and antigen	6	0.156*	0.63	0.71	0.76	0.80	0.84	0.88	0.91	0.94	0.97	1.0	1.03	1.06	1.10	1.14	1.19	1.24	1.31	1.41
	12	0.113	0.72	0.78	0.82	0.85	0.89	0.91	0.93	0.96	0.98	1.0	1.02	1.04	1.08	1.10	1.12	1.18	1.22	1.28
Test for tuberculosis—incubation for one and one-half hours at 37°C.; sixty minutes for hemolysis																				
Complement alone or with antigen	1 to 2	0.125	0.69	0.76	0.80	0.84	0.87	0.90	0.92	0.95	0.97	1.0	1.02	1.05	1.08	1.11	1.15	1.19	1.24	1.32
With serum	1 to 2	0.210†	0.53	0.63	0.70	0.75	0.80	0.84	0.87	0.92	0.96	1.0	1.04	1.09	1.14	1.19	1.26	1.34	1.43	1.58
With serum and antigen	1 to 3	0.156	0.63	0.71	0.76	0.80	0.84	0.88	0.91	0.94	0.97	1.0	1.03	1.06	1.10	1.14	1.19	1.24	1.31	1.41
	6	0.109	0.73	0.79	0.83	0.86	0.89	0.92	0.94	0.96	0.98	1.0	1.03	1.05	1.08	1.10	1.14	1.17	1.21	1.28
	12	0.073	0.81	0.86	0.88	0.91	0.92	0.94	0.96	0.97	0.99	1.0	1.02	1.03	1.05	1.06	1.09	1.11	1.13	1.18

Test for gonococcus infection—incubation for one and one-half hours at 37°C.; fifteen minutes for hemolysis

Complement alone	1 to 2	0.119	0.70	0.77	0.81	0.84	0.87	0.89	0.93	0.94	0.97	1.0	1.02	1.04	1.06	1.09	1.14	1.18	1.22	1.30
With serum and antigen	1 to 3	0.155‡	0.63	0.71	0.76	0.81	0.84	0.88	0.91	0.94	0.97	1.0	1.03	1.06	1.10	1.15	1.19	1.25	1.32	1.43
	4	0.145	0.65	0.72	0.78	0.82	0.86	0.89	0.92	0.94	0.97	1.0	1.03	1.05	1.09	1.13	1.16	1.22	1.28	1.37
	6	0.130	0.68	0.76	0.79	0.83	0.86	0.90	0.92	0.95	0.97	1.0	1.02	1.05	1.07	1.11	1.15	1.20	1.25	1.34
	8	0.110	0.71	0.78	0.82	0.86	0.88	0.90	0.93	0.95	0.97	1.0	1.02	1.03	1.06	1.09	1.11	1.15	1.20	1.26
	12	0.084	0.77	0.82	0.86	0.89	0.91	0.94	0.96	0.97	0.98	1.0	1.02	1.03	1.05	1.07	1.09	1.12	1.15	1.20

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* The $1/n$ value for complement without preliminary incubation and in the absence of serum or antigen when fifteen minutes are allowed for hemolysis is also 0.156; the conversion factors are therefore similar.

† The $1/n$ value for complement without preliminary incubation and in the absence of serum or antigen when sixty minutes are allowed for hemolysis is also 0.210; the conversion factors are therefore similar.

‡ The $1/n$ value for complement with serum is also 0.155; the conversion factors are therefore similar.

‖ The $1/n$ value for complement with cerebrospinal fluid is 0.121. The conversion factors are not used routinely and therefore are not listed. The differences in titers of cerebrospinal fluids that would result from using these factors in place of the ones found with serum were not great enough to make it seem practical to use two separate sets of ratio tables for evaluating results of tests of sera and cerebrospinal fluids.

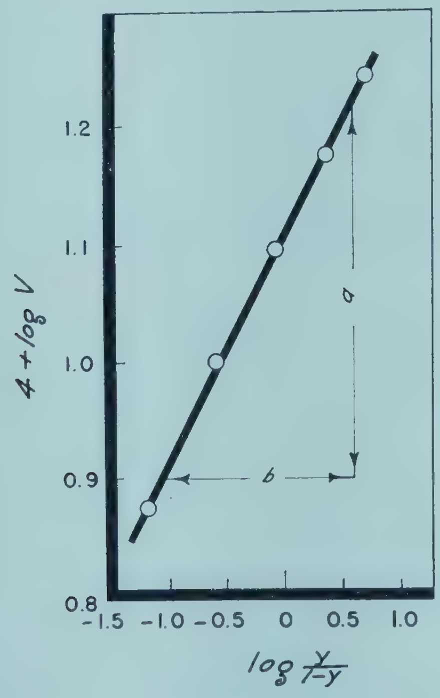
senting 3, 6, and 12 units as used in quantitative tests, determine the 50-per-cent unit values; that is, the conversion factors for different percentages of hemolysis from 5 to 90 at intervals of 5 per cent. These conversion factors may be determined (1) by plotting the amounts of complement (in milliliters) against the degrees of hemolysis obtained in the titrations, fitting, by inspection, a smooth curve to the experimental points in each titration as in the example given in figure 43 and dividing the amount of complement indicated on the curve for a given degree of hemolysis by the amount for 50-per-cent hemolysis; that is, by expressing in terms of 50-per-cent units (column 4 of table 31); or (2) as was done in computing the conversion factors given in table 30 by using the observed fact that if x is the amount of complement required for a given degree of hemolysis y, then the experimental points $\left(\log x, \log \frac{y}{1-y}\right)$ fall approximately on a straight line; that is, $\log x = \log K + 1/n \log \frac{y}{1-y}$ where K is the value of x for $y = 0.5$ and $1/n$ is a parametric constant. This constant is equal to the slope of the line, which appears to vary inversely with K, the amount of complement required for 50-per-cent hemolysis. An evaluation of $1/n$ may be obtained directly by plotting $\log V$, where V is the corresponding volume of complement used experimentally against the logarithm of $\frac{y}{1-y}$ and determining the slope of a fitted straight line as illustrated in figure 50 where $1/n = 0.196$. The conversion factors may be calculated by reading from the fitted line the values for $\log V$ for different values of $\frac{y}{1-y}$ and substracting the value of $\log V$ where $y = 0.5$ (50-per-cent hemolysis), which will give the corresponding values of $\log x$. The corresponding antilogarithms will be the required conversion factors. Table 31 illustrates the calculations for determining conversion factors by both methods. Figure 50 is a logarithmic plot of the same complement titration illustrated in figure 43 (p. 368). Conversion factors as determined by the two methods are essentially the same, but significant deviations from the usual reaction, which may occur in individual titrations, are more readily detected by the second method. Figure 50 also illustrates the method of determining the slope, $1/n$. Further details (5, 8, 9) and alternative methods (41a, 41b) are given in other publications.

Perform titrations sufficient in number to establish accurately new conversion values for use with the new antigen. If reactions in tests with the new antigen when evaluated by the proper conversion factors do not indicate linear relationships between complement and serum, the antigen is unsatisfactory for use in quantitative tests.

TABLE 31

Determination of conversion factors

PER CENT HEMOLYSIS 100 y	COMPLEMENT			$\log \frac{y}{1-y}$	$4 + \log V$		$\log x$	x
	Observed values in ml. $= V$	V read from curve	50% units or conversion factors		Observed	Read from straight line	log V for each per cent hemolysis minus log V for 50% hemolysis	50% units or conversion factors
5		0.00068	0.52	−1.279		0.857	$\bar{1}.752$	0.56
7	0.00075			−1.123	0.8751			
10		0.00079	0.61	−0.954		0.919	$\bar{1}.814$	0.65
20	0.001	0.00096	0.74	−0.602	1.0000	0.987	$\bar{1}.882$	0.76
30		0.00109	0.84	−0.368		1.031	$\bar{1}.926$	0.84
40		0.00120	0.92	−0.176		1.069	$\bar{1}.964$	0.92
45	0.00125			−0.087	1.0969			
50		0.0013	1.0	0		1.105	0	1.0
60		0.00140	1.03	0.176		1.139	0.034	1.08
70	0.0015	0.00151	1.16	0.368	1.1761	1.177	0.072	1.18
80		0.00166	1.28	0.602		1.225	0.120	1.32
85	0.00175			0.753	1.2430			
90		0.00190	1.46	0.954		1.293	0.188	1.54



$$1/n = \frac{a}{b} = 0.196$$

FIG. 50. LOGARITHMIC PLOT OF A COMPLEMENT TITRATION

PREPARATION OF IMMUNE SERUM

TUBERCULOUS IMMUNE SERUM

The standard immune serum that is used to test and control the activity of the tubercle antigens is the serum of a horse immunized to the homologous strain of the tubercle bacillus (27). The serum is dispensed in convenient amounts, sterilized by heating on three successive days for one-half hour at 56°C. and stored in the cold room. Serum that has been stored is reinactivated for one-half hour at 56°C. on the day it is to be used and on subsequent days for five minutes at the same temperature.

ANTIGONOCOCCUS RABBIT SERUM

Healthy normal rabbits are selected for the production of antigonococcus serum. A bleeding is taken from the ear before immunization is begun, and the serum tested for anticomplementary activity. If less than 1.5 units of complement are required to produce 50-per-cent hemolysis in the presence of 0.05 ml. of serum, the animal is considered satisfactory for immunization and the remainder of the normal serum is stored for comparative tests.

For the preparation of the vaccine, cultures of representative strains of gonococci are made on rabbit-blood agar slants (*F43A*). After twenty-four hours' incubation, the growth is washed off the surface of the agar culture with 3–5 ml. of salt solution. The bacterial suspension is transferred to a test tube and the opacity adjusted to that of glass turbidity standard No. 4 (p. 26). For the first injection a suspension heated for one hour at 56°C. is used; unheated culture suspension is injected subsequently. The suspension is injected intravenously as soon as possible after preparation.

Schedule of doses: Heated suspension—0.2 ml., first day. Unheated suspension—0.3 ml., second day; 0.5 ml., fifth and twelfth days; 0.7 ml., fourteenth day; and 1 ml., sixteenth day.

A trial bleeding is taken on the twenty-second day; and, if the serum has a titer of 100 or higher, a large bleeding on the twenty-third day.

The sera are pooled, filtered through a Mandler candle, tested for sterility, and dispensed in 2-ml. amounts in 5-ml. bottles. The sera are inactivated for one-half hour at 56°C. before using and on subsequent days for five minutes at the same temperature.

TITRATION OF IMMUNE SERA

The titer of the immune sera or of patients' sera used in standardization of antigens or in controls is determined as indicated on p. 438, "Determination of Titers Greater than 10." The titers of tuberculous immune horse sera usually range from 200 to 400; those of antigonococcus rabbit sera, from 150 to 450.

OPENING AND NUMBERING SPECIMENS

The specimens, usually consisting of 5–10 ml. of clotted blood or cerebrospinal fluid, are received in the outfits provided by the laboratory.

The dry sterile tube and Petroff bleeding needle in these outfits are especially recommended for securing satisfactory blood specimens. If syringes are used they should be clean, sterile, and dry.

Open and number only one specimen at a time. Stamp the serial number of each on two gummed labels—one to be placed on the specimen tube and one on the tube that will receive the serum after its removal from the clot—and then on the history form.

The serial numbers are stamped also on the accession sheet that is later used for recording the readings of the tests.

Use separate series of accession numbers for blood and cerebrospinal fluid. Place the specimen tubes consecutively as stamped in racks with holes for ten tubes. A specimen with serial number ending in "1" is put in the first place at the left and so on in order, so that a specimen with a number ending in a given figure may always be found in a certain position in the rack. If a specimen tube is found broken on arrival, number an empty tube correspondingly and substitute it in the rack. Cover all specimen tubes with loosely fitting metal caps before centrifuging (42).

Centrifugation of specimens in open tubes results in significant increases, sometimes up to 10 per cent, in their protein concentration.

Record any unusual appearance of the specimen on the history.

For the guidance of technicians, a list is prepared each day, containing the first and last serial numbers of the specimens received, the serial numbers of cerebrospinal fluids, specimens on which telegraphed or "rush" reports are requested, specimens that are being retested or have been sent to another department for other tests, and those which for any reason are unsatisfactory for examination.

PREPARATION OF SPECIMENS

BLOOD SERUM

After centrifugation of the specimens, transfer the clear serum from each to a clean tube numbered correspondingly. Use for this purpose an Asepto pipette fitted with a rubber bulb, being careful not to disturb the cells. Specimens should be capped before inactivation and during storage.

Inactivate specimens of blood serum in a water bath at 56°C. (43) for one-half hour on the day they are to be tested. If a serum is to be retested twenty-four hours or more after the original half-hour inactivation, reinactivate it for five minutes at 56°C.

If the temperature of the inactivating bath should rise above 56.5°C. or fall below 55.5°C., obtain a new portion of each serum from the original clot if possible.

If a specimen has been delayed in transit or if any serum is unusual in appearance, for example, is found to be hemolyzed, bile-stained, chylous, or markedly discolored, note the fact on the corresponding history blank. If a serum is badly hemolyzed, list it among those unsatisfactory for examination and discard it.

A color standard for eliminating badly hemolyzed specimens is prepared by laking 0.15 ml. of washed, packed sheep cells in 0.85 ml. of distilled water. Specimens containing an amount of hemoglobin greater than that of the standard are not examined; those with a quantity less than this but more than that indicated by a standard prepared by laking 0.05 ml. of washed, packed sheep cells in 1.95 ml. of distilled water, are examined but the report indicates that because the specimen was hemolyzed the results may require confirmation.

CEREBROSPINAL FLUID

Note on the back of the history slip the appearance of each cerebrospinal fluid; that is, turbidity, discoloration, presence of hemoglobin, blood cells, or sediment. It is not satisfactory for tests if it is purulent or contains fibrin or more than a slight amount of blood. Refer all fluids for microscopic examination of the sediment. Centrifuge all cerebrospinal fluids in capped tubes for twenty minutes at 1800 r.p.m., withdraw the supernatant fluid of each to a clean tube, and record its appearance. Three milliliters of supernatant cerebrospinal fluid should be available for serologic and other tests.

If cerebrospinal fluids contain blood and are to be tested, inactivate them for one-half hour at 56°C., on the day they are prepared and allow to stand overnight in the cold room so that the carbon dioxide content will correspond to that of unheated specimens.

The precautions usually taken in handling material that may be infective should be observed with cerebrospinal fluids.

Except when immediate reports are requested, specimens of blood and of cerebrospinal fluid for the complement-fixation test are not examined until the next working day following their arrival at the laboratory. As soon as the preparation of each lot of specimens is completed, at noon or night, the tubes are capped and stored in the cold room.

QUANTITATIVE COMPLEMENT-FIXATION TEST

DETERMINATION OF TITERS UP TO 10

Perform the tests for syphilis, tuberculosis, and gonococcus infection as outlined in tables 32, 33, and 34. In the first and last sets of tests that are made on the same day include the following controls. Test two reacting sera of different titers or two different dilutions of immune serum and a known normal serum to control the reactivity of the antigen preparations. In the case of normal serum, omit the test with 6 and 12 units of complement but add a test with 2 units. Prepare controls of the anticomplementary activity of each dilution of antigen with 1 and 2 units of complement; also controls of 1 and 2 units of complement and one of sensitized cells alone.

TABLE 32

The quantitative complement-fixation test for syphilis

TUBE NO.	PATIENT'S SERUM*	ANTIGEN DILUTION† SELECTED FOR USE IN TESTS OF SERA WITH COMPLEMENT			COMPLEMENT DILUTION CONTAINING				SALT SOLUTION	FIXATION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
		3 units	6 units	12 units	1 unit	3 units	6 units	12 units				
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*		*ml.*	
1	0.05				0.1				0.15	4 hrs. at 3°–6°C.	0.2	15 minutes at 37°C.
2	0.05				0.1				0.15		0.2	
3	0.05				0.2				0.05		0.2	
4	0.05	0.1				0.1			0.05		0.2	
5	0.05		0.1				0.1		0.05		0.2	
6	0.05			0.1				0.1	0.05		0.2	

* Or 0.2 ml. of cerebrospinal fluid; in such tests add salt solution in amounts to make the total volume before fixation 0.4 ml.

† Cardiolipin antigen diluted to 1:133, 1:67, and 1:33; cholesterolized tissue extract diluted to 1:100, 1:50, and 1:25 for use respectively with 3, 6, and 12 units of complement in the test of serum. In the test of cerebrospinal fluid the dilutions of cardiolipin so used would be 1:266, 1:90, and 1:33, and of cholesterolized tissue extract 1:200, 1:100, and 1:33.

TABLE 33

The quantitative complement-fixation test for tuberculosis

TUBE NO.	PATIENT'S SERUM*	ANTIGEN (MAXIMALLY REACTIVE DOSE)	COMPLEMENT DILUTION CONTAINING				SALT SOLUTION	FIXATION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
			1 unit	3 units	6 units	12 units				
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*		*ml.*	
1	0.05		0.1				0.15	1½ hrs. at 37°C.	0.2	60 minutes at 37°C.
2	0.05		0.1				0.15		0.2	
3	0.05		0.2				0.05		0.2	
4	0.05	0.1		0.1			0.05		0.2	
5	0.05	0.1			0.1		0.05		0.2	
6	0.05	0.1				0.1	0.05		0.2	

* In tests of the cerebrospinal fluid from a limited number of patients with tuberculous meningitis, no reactions have been obtained.

With the aqueous extract used as antigen in the test for tuberculosis, include tests of 0.1-ml. amounts of the undiluted isotonic extract with 2 and 3 units of complement as an added control of the anticomplementary properties.

In complement controls for the test with gonococcus antigens, include 1, 1.5, and 2 units of complement.

TABLE 34

The quantitative complement-fixation test with gonococcus antigens

TUBE NO.	PATIENT'S SERUM	COMPLEMENT DILUTION CONTAINING*								ANTIGEN MAXIMALLY REACTIVE DOSE	SALT SOLUTION	FIXATION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
		1 unit	1 unit	2 units	2 units	4 units	4 units	8 units	12 units					
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*		*ml.*	
1	0.05	0.1									0.15	1½ hrs. at 37°C.	0.2	15 minutes at 37°C.
2	0.05		0.15								0.1		0.2	
3	0.05			0.1							0.15		0.2	
4	0.05			0.1						0.1	0.05		0.2	
5	0.05				0.15					0.1	0		0.2	
6	0.05					0.1				0.1	0.05		0.2	
7	0.05						0.15			0.1	0		0.2	
8	0.05							0.1		0.1	0.05		0.2	
9	0.05								0.1	0.1	0.05		0.2	

* These quantities of the different complement dilutions represent 1, 1.5, 2, 3, 4, 6, 8, and 12 units.

Reading and Recording Results

Read each test by comparison with the color standards; record the percentage of hemolysis in each tube.

When 50 or more specimens that react in the quantitative test are examined on a given day, the percentage of hemolysis in serum controls of nonreacting specimens may not need to be recorded since no titers are determined with these sera. These serum controls, however, in addition to being used in computing titers are of value as a check in the adjustment of the hemolytic system and also may indicate improper inactivation of specimens. Hence the total number recorded each day must be sufficient to provide this essential information. Likewise, any deviation from the usual reaction in serum controls of nonreacting specimens which might suggest improper handling or testing of certain groups of specimens must be noted.

Repeat tests with specimens that inhibit complement to an unusual degree, namely, when less than 55-per-cent hemolysis occurs in the serum control with 2 units of complement.

In the repeated test include controls with larger amounts of complement and tests with smaller amounts of serum or cerebrospinal fluid.

Repeat also any tests that indicate disagreement with the results of other serologic tests, irregular reactions, or any technical inaccuracies.

Computation of Titer

Evaluate tests with the different antigens by referring to charts (tables 35–46) giving the ratio:

$$\frac{\text{(amount of complement required for 50-per-cent hemolysis with serum and antigen)}}{\text{(amount of complement required for 50-per-cent hemolysis with serum alone)}}$$

These ratios are computed for all combinations of reactions that may occur in the serum controls and in the tests, as indicated in the following example: Suppose 0.0013 ml. is 1 unit of complement giving 50-per-cent hemolysis under the conditions of the test for syphilis; if the hemolysis is 20 per cent in the serum control with 1 unit of complement, determine the amount required for 50-per-cent hemolysis by dividing 0.0013 ml. by 0.76, the conversion factor for 20-per-cent hemolysis according to table 30 (p. 416).

$$\frac{0.0013 \text{ ml.}}{0.76} = 0.00171 \text{ ml.}$$

If the hemolysis is 30 per cent in the test of the serum with antigen with 6 units of complement, determine the amount required for 50-per-cent hemolysis by dividing 0.0078 ml. by 0.88, the conversion factor for 30-per-cent hemolysis in the third row of table 30.

$$\frac{0.0078 \text{ ml.}}{0.88} = 0.00886 \text{ ml.}$$

Then the ratio of the amount of complement required for 50-per-cent hemolysis in the presence of serum and antigen to that required for 50-per-cent hemolysis in the presence of serum alone is, in this case,

$$\frac{0.00886 \text{ ml.}}{0.00171 \text{ ml.}} = 5.2$$

The conversion factors for use in tests with different antigens vary somewhat as indicated in table 30, but they are used in a similar manner in computing titers.

Ratios for any combination of reactions can be read directly on comple-

TABLE 35

Tabulation of ratios or titers of reactions with present antigen and three units of complement in the test for syphilis

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	2.79	2.47	2.28	2.15	2.06	1.96	1.87	1.80	1.73	1.66	1.60	1.53	1.47	1.41	1.34	1.26	1.17	1.07
10	3.24	2.88	2.65	2.49	2.37	2.27	2.18	2.07	2.00	1.92	1.85	1.77	1.71	1.63	1.55	1.46	1.36	1.24
15	3.56	3.15	2.92	2.74	2.63	2.49	2.39	2.29	2.20	2.11	2.03	1.95	1.87	1.79	1.70	1.60	1.50	1.36
20	3.80	3.38	3.13	2.94	2.80	2.67	2.55	2.45	2.35	2.26	2.17	2.08	2.00	1.91	1.82	1.72	1.60	1.46
25	4.03	3.59	3.31	3.12	2.97	2.83	2.72	2.60	2.49	2.40	2.30	2.21	2.12	2.03	1.92	1.83	1.70	1.55
30	4.25	3.76	3.49	3.27	3.13	2.98	2.85	2.74	2.63	2.52	2.43	2.33	2.24	2.14	2.03	1.92	1.79	1.63
35	4.44	3.95	3.64	3.42	3.26	3.13	2.99	2.86	2.75	2.64	2.54	2.44	2.33	2.24	2.12	2.00	1.86	1.70
40	4.63	4.11	3.78	3.57	3.40	3.25	3.12	2.99	2.86	2.75	2.65	2.54	2.43	2.32	2.22	2.09	1.94	1.77
45	4.82	4.28	3.94	3.72	3.55	3.39	3.24	3.10	2.99	2.86	2.75	2.65	2.53	2.42	2.30	2.18	2.03	1.85
50	5.02	4.45	4.12	3.88	3.69	3.52	3.37	3.24	3.10	3.00	2.86	2.75	2.64	2.52	2.40	2.26	2.12	1.92
55	5.24	4.62	4.29	4.04	3.84	3.67	3.52	3.37	3.24	3.12	2.99	2.86	2.75	2.63	2.50	2.36	2.20	2.00
60	5.44	4.83	4.45	4.19	4.00	3.81	3.65	3.50	3.36	3.23	3.11	2.99	2.86	2.73	2.60	2.45	2.28	2.08
65	5.67	5.04	4.64	4.40	4.18	3.98	3.82	3.65	3.52	3.38	3.24	3.12	2.99	2.85	2.71	2.56	2.40	2.18
70	5.94	5.30	4.87	4.58	4.38	4.19	4.00	3.83	3.68	3.53	3.40	3.26	3.13	2.98	2.84	2.69	2.50	2.27
75	6.25	5.55	5.13	4.83	4.59	4.40	4.20	4.03	3.88	3.72	3.57	3.43	3.30	3.14	2.98	2.82	2.63	2.40
80	6.65	5.87	5.44	5.12	4.86	4.64	4.45	4.27	4.10	3.94	3.79	3.64	3.48	3.33	3.15	2.98	2.79	2.54
85	7.15	6.30	5.82	5.48	5.23	4.97	4.76	4.56	4.40	4.21	4.05	3.89	3.74	3.57	3.39	3.20	2.98	2.72
90	7.75	6.90	6.35	6.00	5.71	5.46	5.23	5.00	4.81	4.62	4.44	4.25	4.09	3.90	3.72	3.51	3.27	2.97

A small correction, usually not above 7 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with two units of complement, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 36

Tabulation of ratios or titers of reactions with present antigen and six units of complement in the test for syphilis

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	4.97	4.58	4.32	4.13	3.97	3.82	3.69	3.55	3.44	3.34	3.23	3.11	3.00	2.88	2.74	2.60	2.43	2.22
10	5.83	5.35	5.00	4.78	4.58	4.43	4.27	4.12	3.98	3.85	3.73	3.60	3.47	3.34	3.18	3.02	2.82	2.56
15	6.40	5.84	5.52	5.25	5.05	4.85	4.69	4.52	4.37	4.23	4.10	3.95	3.80	3.66	3.50	3.31	3.10	2.81
20	6.80	6.25	5.88	5.63	5.43	5.20	5.01	4.84	4.67	4.53	4.38	4.23	4.07	3.92	3.74	3.54	3.31	3.02
25	7.25	6.65	6.25	5.95	5.73	5.52	5.33	5.13	4.95	4.80	4.63	4.49	4.32	4.14	3.95	3.75	3.50	3.20
30	7.70	7.00	6.60	6.25	6.05	5.79	5.60	5.40	5.23	5.05	4.88	4.71	4.55	4.38	4.17	3.95	3.70	3.36
35	8.00	7.30	6.85	6.55	6.30	6.10	5.85	5.65	5.49	5.32	5.13	4.94	4.75	4.56	4.36	4.13	3.85	3.52
40	8.30	7.65	7.20	6.80	6.60	6.30	6.15	5.89	5.68	5.52	5.34	5.15	4.96	4.76	4.54	4.30	4.02	3.67
45	8.70	7.90	7.45	7.15	6.80	6.60	6.30	6.15	5.92	5.74	5.56	5.38	5.17	4.95	4.73	4.48	4.19	3.82
50	9.05	8.25	7.75	7.40	7.20	6.80	6.65	6.35	6.20	6.00	5.76	5.59	5.39	5.16	4.93	4.65	4.37	3.98
55	9.40	8.60	8.10	7.75	7.45	7.20	6.90	6.70	6.40	6.25	6.05	5.81	5.62	5.40	5.14	4.85	4.54	4.13
60	9.80	8.95	8.40	8.05	7.75	7.45	7.20	6.90	6.70	6.50	6.25	6.10	5.81	5.60	5.35	5.05	4.71	4.30
65	10.3	9.35	8.75	8.35	8.10	7.75	7.50	7.25	7.00	6.75	6.60	6.30	6.15	5.84	5.59	5.29	4.93	4.50
70	10.7	9.75	9.20	8.80	8.45	8.20	7.80	7.60	7.30	7.10	6.80	6.65	6.35	6.15	5.83	5.54	5.16	4.70
75	11.3	10.3	9.70	9.25	8.85	8.55	8.20	7.90	7.70	7.45	7.20	6.95	6.75	6.40	6.15	5.80	5.44	4.95
80	11.9	10.9	10.3	9.80	9.40	9.10	8.75	8.45	8.20	7.85	7.70	7.40	7.15	6.80	6.50	6.20	5.74	5.25
85	12.7	11.7	11.0	10.5	10.2	9.70	9.35	9.05	8.75	8.50	8.20	7.85	7.65	7.30	6.95	6.60	6.20	5.62
90	14.0	12.8	12.0	11.5	11.1	10.7	10.3	9.90	9.60	9.25	8.95	8.65	8.30	8.00	7.70	7.25	6.75	6.20

A small correction, usually not above 7 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50 per cent hemolysis (44).

To determine the value of a test on the basis of the serum control with two units of complement, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 37

Tabulation of ratios or titers of reactions with present antigen and twelve units of complement in the test for syphilis

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	8.30	7.95	7.75	7.55	7.35	7.25	7.10	6.90	6.80	6.65	6.50	6.35	6.20	6.05	5.82	5.61	5.32	4.93
10	9.70	9.25	8.95	8.75	8.55	8.35	8.20	8.00	7.80	7.70	7.55	7.35	7.20	7.00	6.75	6.50	6.20	5.70
15	10.6	10.2	9.85	9.60	9.35	9.20	9.00	8.80	8.65	8.45	8.25	8.10	7.90	7.70	7.45	7.15	6.75	6.25
20	11.4	10.9	10.6	10.3	10.1	9.80	9.70	9.45	9.25	9.05	8.80	8.70	8.45	8.20	7.90	7.70	7.25	6.70
25	12.0	11.6	11.2	10.9	10.7	10.5	10.3	10.0	9.80	9.60	9.35	9.20	8.95	8.70	8.40	8.10	7.70	7.15
30	12.6	12.2	11.7	11.5	11.3	11.0	10.7	10.5	10.3	10.2	9.85	9.70	9.45	9.20	8.85	8.50	8.10	7.50
35	13.3	12.7	12.3	12.0	11.7	11.5	11.3	11.1	10.8	10.6	10.3	10.2	9.85	9.60	9.25	8.90	8.40	7.80
40	13.8	13.3	12.8	12.5	12.3	12.0	11.7	11.5	11.3	11.1	10.8	10.6	10.3	10.0	9.70	9.30	8.75	8.20
45	14.4	13.8	13.4	13.0	12.7	12.5	12.3	12.0	11.7	11.5	11.3	11.0	10.7	10.4	10.1	9.70	9.15	8.50
50	15.0	14.4	14.0	13.5	13.3	13.0	12.7	12.5	12.2	12.0	11.7	11.5	11.2	10.8	10.5	10.1	9.55	8.85
55	15.6	15.0	14.5	14.2	13.8	13.6	13.3	13.0	12.7	12.5	12.2	11.9	11.6	11.3	11.0	10.5	9.95	9.25
60	16.3	15.6	15.1	14.7	14.4	14.1	13.8	13.5	13.3	12.9	12.6	12.4	12.1	11.7	11.4	11.0	10.3	9.60
65	17.0	16.3	15.7	15.4	15.0	14.7	14.4	14.2	13.8	13.5	13.3	13.0	12.6	12.3	11.8	11.4	10.8	10.0
70	17.7	17.0	16.5	16.1	15.7	15.5	15.1	14.7	14.5	14.2	13.8	13.5	13.2	12.8	12.4	12.0	11.3	10.5
75	18.6	17.8	17.3	16.9	16.5	16.2	15.8	15.5	15.2	14.9	14.6	14.3	13.9	13.5	13.0	12.5	11.8	11.1
80	19.7	18.9	18.4	17.9	17.5	17.2	16.8	16.5	16.2	15.8	15.5	15.2	14.7	14.3	13.8	13.3	12.6	11.7
85	21.3	20.3	19.6	19.2	18.7	18.4	18.0	17.6	17.3	17.0	16.5	16.2	15.8	15.3	14.8	14.3	13.5	12.5
90	23.3	22.3	21.6	21.1	20.6	20.2	19.7	19.3	18.9	18.5	18.1	17.7	17.3	16.8	16.3	15.6	14.8	13.7

A small correction, usually not above 7 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in the titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with two units of complement, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 38

Tabulation of ratios or titers of reactions with present antigen and three units of complement in the test for tuberculosis

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	2.34	2.16	2.05	1.96	1.88	1.82	1.77	1.71	1.67	1.61	1.57	1.52	1.47	1.42	1.37	1.32	1.25	1.16
10	2.75	2.52	2.40	2.29	2.21	2.13	2.07	2.00	1.95	1.90	1.84	1.78	1.73	1.67	1.60	1.54	1.46	1.36
15	3.02	2.79	2.64	2.52	2.43	2.35	2.27	2.20	2.14	2.08	2.02	1.95	1.90	1.83	1.76	1.69	1.60	1.50
20	3.26	3.00	2.84	2.72	2.62	2.52	2.44	2.38	2.31	2.25	2.18	2.11	2.04	1.98	1.90	1.82	1.73	1.61
25	3.46	3.20	3.02	2.89	2.78	2.69	2.60	2.51	2.45	2.38	2.31	2.24	2.18	2.10	2.02	1.93	1.83	1.71
30	3.67	3.37	3.18	3.05	2.93	2.83	2.73	2.66	2.59	2.51	2.44	2.37	2.29	2.22	2.13	2.04	1.93	1.81
35	3.83	3.53	3.33	3.19	3.07	2.98	2.88	2.79	2.71	2.63	2.57	2.48	2.40	2.32	2.23	2.13	2.03	1.88
40	4.01	3.70	3.50	3.34	3.21	3.11	3.00	2.92	2.84	2.76	2.68	2.60	2.51	2.43	2.33	2.23	2.12	1.98
45	4.20	3.86	3.65	3.49	3.36	3.25	3.14	3.05	2.96	2.88	2.80	2.71	2.63	2.53	2.45	2.34	2.22	2.07
50	4.36	4.01	3.80	3.62	3.50	3.38	3.28	3.18	3.09	3.00	2.91	2.82	2.73	2.63	2.53	2.43	2.30	2.14
55	4.55	4.20	3.97	3.78	3.66	3.53	3.42	3.32	3.22	3.13	3.03	2.95	2.86	2.76	2.66	2.54	2.41	2.24
60	4.77	4.40	4.13	3.97	3.81	3.70	3.59	3.47	3.37	3.28	3.18	3.08	2.98	2.88	2.77	2.66	2.51	2.34
65	4.96	4.56	4.33	4.13	3.97	3.84	3.72	3.62	3.51	3.42	3.31	3.20	3.12	3.00	2.90	2.77	2.63	2.44
70	5.22	4.81	4.53	4.35	4.19	4.05	3.91	3.80	3.70	3.60	3.49	3.38	3.27	3.16	3.04	2.91	2.76	2.57
75	5.50	5.05	4.80	4.56	4.40	4.27	4.12	4.00	3.89	3.78	3.68	3.55	3.45	3.32	3.20	3.07	2.91	2.70
80	5.83	5.40	5.09	4.86	4.69	4.52	4.40	4.25	4.12	4.02	3.90	3.78	3.67	3.53	3.41	3.26	3.10	2.88
85	6.25	5.79	5.48	5.25	5.02	4.87	4.70	4.57	4.45	4.33	4.20	4.06	3.94	3.80	3.66	3.50	3.32	3.10
90	6.90	6.33	6.02	5.75	5.53	5.36	5.18	5.02	4.88	4.75	4.60	4.48	4.33	4.19	4.02	3.85	3.66	3.40

A small correction, usually not above 7 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with two units of complement, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 39

Tabulation of ratios or titers of reactions with present antigen and six units of complement in the test for tuberculosis

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	4.34	4.04	3.87	3.73	3.63	3.55	3.47	3.39	3.31	3.23	3.17	3.10	3.02	2.95	2.85	2.76	2.63	2.47
10	5.07	4.73	4.52	4.49	4.26	4.15	4.05	3.96	3.88	3.79	3.70	3.61	3.53	3.43	3.33	3.23	3.08	2.89
15	5.60	5.22	4.98	4.82	4.67	4.55	4.46	4.36	4.27	4.16	4.07	3.98	3.89	3.77	3.67	3.55	3.40	3.18
20	6.00	5.61	5.38	5.20	5.05	4.90	4.80	4.70	4.60	4.50	4.40	4.28	4.19	4.08	3.95	3.81	3.65	3.42
25	6.40	5.95	5.70	5.51	5.36	5.21	5.10	4.98	4.87	4.76	4.65	4.55	4.44	4.33	4.20	4.06	3.87	3.63
30	6.75	6.27	6.05	5.81	5.65	5.51	5.39	5.27	5.15	5.03	4.92	4.80	4.68	4.56	4.43	4.29	4.08	3.84
35	7.10	6.60	6.28	6.10	5.91	5.77	5.65	5.52	5.40	5.28	5.16	5.03	4.90	4.78	4.63	4.48	4.29	4.02
40	7.43	6.90	6.60	6.38	6.20	6.05	5.90	5.77	5.65	5.53	5.40	5.28	5.15	5.00	4.85	4.70	4.48	4.21
45	7.75	7.22	6.90	6.70	6.48	6.28	6.20	6.02	5.90	5.76	5.62	5.50	5.38	5.23	5.09	4.90	4.67	4.40
50	8.03	7.50	7.20	6.90	6.75	6.55	6.40	6.25	6.15	6.00	5.85	5.70	5.59	5.43	5.29	5.10	4.86	4.55
55	8.40	7.80	7.50	7.24	7.05	6.87	6.70	6.60	6.40	6.25	6.12	5.98	5.83	5.68	5.52	5.35	5.10	4.77
60	8.77	8.15	7.80	7.60	7.37	7.20	7.00	6.83	6.70	6.55	6.40	6.25	6.10	5.92	5.75	5.57	5.32	4.98
65	9.15	8.55	8.15	7.85	7.70	7.50	7.28	7.17	7.00	6.80	6.66	6.50	6.35	6.20	6.00	5.80	5.54	5.20
70	9.63	9.00	8.60	8.27	8.05	7.85	7.70	7.50	7.30	7.15	7.00	6.80	6.70	6.50	6.30	6.10	5.80	5.46
75	10.1	9.45	9.10	8.75	8.50	8.25	8.10	7.90	7.73	7.60	7.40	7.20	7.05	6.83	6.67	6.44	6.15	5.75
80	10.8	10.1	9.60	9.25	9.00	8.76	8.60	8.40	8.22	8.00	7.80	7.70	7.50	7.27	7.10	6.80	6.55	6.10
85	11.6	10.8	10.3	10.0	9.73	9.45	9.25	9.05	8.82	8.65	8.45	8.22	8.05	7.83	7.60	7.35	7.00	6.60
90	12.8	11.9	11.4	11.0	10.7	10.4	10.2	9.95	9.74	9.50	9.27	9.10	8.85	8.60	8.38	8.10	7.72	7.25

A small correction, usually not above 7 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with two units of complement, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 40

Tabulation of ratios or titers of reactions with present antigen and twelve units of complement in the test for tuberculosis

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	7.60	7.35	7.20	7.03	6.90	6.80	6.74	6.65	6.55	6.45	6.33	6.25	6.18	6.07	5.92	5.79	5.60	5.38
10	8.88	8.60	8.40	8.20	8.10	8.00	7.85	7.75	7.68	7.56	7.45	7.30	7.23	7.10	6.90	6.80	6.55	6.27
15	9.80	9.49	9.25	9.10	8.90	8.78	8.70	8.55	8.45	8.28	8.15	8.03	7.90	7.78	7.63	7.49	7.23	6.90
20	10.6	10.2	9.95	9.80	9.60	9.50	9.35	9.20	9.10	8.95	8.80	8.65	8.55	8.40	8.20	8.00	7.75	7.45
25	11.2	10.8	10.6	10.3	10.2	10.1	9.95	9.77	9.60	9.50	9.35	9.20	9.10	8.90	8.73	8.55	8.23	7.90
30	11.8	11.4	11.2	11.0	10.8	10.6	10.4	10.3	10.2	10.0	9.90	9.75	9.60	9.40	9.20	9.00	8.70	8.32
35	12.4	12.0	11.7	11.5	11.3	11.2	11.0	10.8	10.7	10.5	10.4	10.3	10.0	9.85	9.65	9.40	9.10	8.70
40	13.0	12.5	12.2	12.0	11.8	11.7	11.5	11.3	11.2	11.0	10.8	10.7	10.5	10.3	10.1	9.80	9.55	9.15
45	13.6	13.2	12.8	12.5	12.3	12.2	12.0	11.8	11.7	11.5	11.3	11.1	11.0	10.8	10.6	10.3	10.0	9.55
50	14.1	13.7	13.3	13.0	12.8	12.6	12.5	12.3	12.1	12.0	11.8	11.6	11.4	11.2	11.0	10.7	10.3	9.90
55	14.8	14.3	13.9	13.6	13.4	13.3	13.1	12.9	12.7	12.5	12.3	12.1	11.9	11.7	11.5	11.2	10.8	10.3
60	15.4	14.9	14.6	14.3	14.0	13.8	13.7	13.5	13.2	13.1	12.8	12.7	12.4	12.2	12.0	11.7	11.3	10.8
65	16.1	15.5	15.2	14.8	14.7	14.4	14.3	14.0	13.8	13.7	13.4	13.2	13.0	12.8	12.5	12.2	11.8	11.3
70	16.9	16.3	16.0	15.6	15.3	15.2	15.0	14.8	14.5	14.3	14.2	13.8	13.7	13.4	13.2	12.8	12.4	11.8
75	17.8	17.2	16.8	16.4	16.2	16.0	15.7	15.5	15.3	15.1	14.9	14.7	14.4	14.2	13.8	13.5	13.1	12.5
80	18.9	18.3	17.8	17.4	17.2	17.0	16.7	16.5	16.3	16.0	15.8	15.5	15.3	15.0	14.7	14.4	13.9	13.3
85	20.3	19.7	19.2	18.8	18.5	18.2	18.0	17.8	17.5	17.3	17.0	16.7	16.5	16.2	15.8	15.4	15.0	14.3
90	22.3	21.7	21.2	20.7	20.3	20.1	19.8	19.5	19.2	18.9	18.7	18.4	18.1	17.8	17.4	17.0	16.4	15.7

A small correction, usually not above 7 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with two units of complement, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 41

Tabulation of ratios or titers of reactions with present antigen and two units of complement in the test for gonococcus infection

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	2.0	1.8	1.7	1.6	1.5	1.5	1.4	1.4	1.3	1.3	1.2	1.2	1.2	1.1	1.1	1.0	1.0	0.9
10	2.2	2.0	1.9	1.8	1.7	1.6	1.6	1.5	1.5	1.4	1.4	1.3	1.3	1.2	1.2	1.2	1.1	1.0
15	2.4	2.1	2.0	1.9	1.8	1.7	1.7	1.6	1.6	1.5	1.5	1.4	1.4	1.3	1.3	1.2	1.2	1.1
20	2.5	2.2	2.1	2.0	1.9	1.8	1.8	1.7	1.7	1.6	1.6	1.5	1.5	1.4	1.4	1.3	1.2	1.1
25	2.6	2.4	2.2	2.1	2.0	1.9	1.9	1.8	1.8	1.7	1.6	1.6	1.5	1.5	1.4	1.4	1.3	1.2
30	2.7	2.4	2.3	2.2	2.1	2.0	1.9	1.9	1.8	1.8	1.7	1.6	1.6	1.5	1.5	1.4	1.3	1.2
35	2.8	2.5	2.4	2.3	2.2	2.1	2.0	1.9	1.9	1.8	1.8	1.7	1.6	1.6	1.5	1.5	1.4	1.3
40	2.9	2.6	2.5	2.3	2.2	2.1	2.1	2.0	1.9	1.9	1.8	1.8	1.7	1.6	1.6	1.5	1.4	1.3
45	3.0	2.7	2.5	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.9	1.8	1.8	1.7	1.6	1.5	1.5	1.4
50	3.1	2.8	2.6	2.5	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.9	1.8	1.8	1.7	1.6	1.5	1.4
55	3.2	2.9	2.7	2.6	2.4	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.9	1.8	1.7	1.7	1.6	1.5
60	3.3	3.0	2.8	2.6	2.5	2.4	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.9	1.8	1.7	1.6	1.5
65	3.4	3.1	2.9	2.7	2.6	2.5	2.4	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.8	1.8	1.7	1.6
70	3.5	3.2	3.0	2.8	2.7	2.6	2.5	2.4	2.4	2.3	2.2	2.1	2.1	2.0	1.9	1.8	1.7	1.6
75	3.7	3.3	3.1	2.9	2.8	2.7	2.6	2.5	2.5	2.4	2.3	2.2	2.2	2.1	2.0	1.9	1.8	1.7
80	3.9	3.4	3.2	3.1	2.9	2.8	2.7	2.6	2.6	2.5	2.4	2.3	2.3	2.2	2.1	2.0	1.9	1.8
85	4.1	3.6	3.4	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.5	2.4	2.3	2.2	2.1	2.0	1.9
90	4.4	3.9	3.6	3.5	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.5	2.4	2.3	2.1	2.0

A small correction value, not over 9 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with 1.5 units of complement, divide by 1.5 or on the basis of control with two units, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 42

Tabulation of ratios or titers of reactions with present antigen and three units of complement in the test for gonococcus infection

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	2.9	2.7	2.5	2.4	2.3	2.2	2.1	2.0	2.0	1.9	1.9	1.8	1.8	1.7	1.6	1.6	1.5	1.4
10	3.3	3.0	2.8	2.6	2.5	2.4	2.4	2.3	2.2	2.2	2.1	2.0	2.0	1.9	1.8	1.8	1.7	1.5
15	3.5	3.2	3.0	2.9	2.7	2.6	2.5	2.5	2.4	2.3	2.3	2.2	2.1	2.0	2.0	1.9	1.8	1.7
20	3.7	3.4	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.4	2.3	2.2	2.2	2.1	2.0	1.9	1.8
25	3.9	3.5	3.3	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.5	2.4	2.3	2.2	2.2	2.1	2.0	1.8
30	4.0	3.6	3.4	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.6	2.5	2.4	2.3	2.2	2.2	2.0	1.9
35	4.2	3.8	3.5	3.4	3.2	3.1	3.0	2.9	2.8	2.7	2.7	2.6	2.5	2.4	2.3	2.2	2.1	2.0
40	4.3	3.9	3.6	3.5	3.3	3.2	3.1	3.0	2.9	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.0
45	4.4	4.0	3.8	3.6	3.4	3.3	3.2	3.1	3.0	2.9	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.1
50	4.6	4.2	3.9	3.7	3.6	3.4	3.3	3.2	3.1	3.0	3.0	2.9	2.8	2.7	2.6	2.5	2.3	2.2
55	4.7	4.3	4.0	3.8	3.7	3.5	3.4	3.3	3.2	3.1	3.0	2.9	2.9	2.8	2.6	2.5	2.4	2.2
60	4.9	4.4	4.2	4.0	3.8	3.7	3.5	3.4	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.3
65	5.0	4.6	4.3	4.1	3.9	3.8	3.6	3.5	3.4	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.4
70	5.2	4.7	4.4	4.2	4.1	3.9	3.8	3.7	3.5	3.4	3.3	3.2	3.1	3.0	2.9	2.8	2.7	2.5
75	5.4	4.9	4.6	4.4	4.2	4.1	3.9	3.8	3.7	3.6	3.5	3.4	3.3	3.1	3.0	2.9	2.8	2.6
80	5.7	5.1	4.8	4.6	4.4	4.3	4.1	4.0	3.9	3.7	3.6	3.5	3.4	3.3	3.2	3.1	2.9	2.7
85	6.0	5.4	5.1	4.8	4.7	4.5	4.3	4.2	4.1	4.0	3.8	3.7	3.6	3.5	3.3	3.2	3.0	2.8
90	6.4	5.8	5.5	5.2	5.0	4.8	4.6	4.5	4.4	4.2	4.1	4.0	3.9	3.7	3.6	3.4	3.3	3.0

A small correction value, not over 9 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with 1.5 units of complement, divide by 1.5 or on the basis of control with two units, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 43

Tabulation of ratios or titers of reactions with present antigen and four units of complement in the test for gonococcus infection

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	3.7	3.4	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.5	2.5	2.4	2.3	2.2	2.1	2.0	1.9
10	4.2	3.8	3.6	3.4	3.3	3.2	3.1	3.0	2.9	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.0
15	4.5	4.1	3.8	3.7	3.6	3.4	3.3	3.2	3.2	3.1	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.2
20	4.7	4.3	4.1	3.9	3.8	3.6	3.5	3.4	3.4	3.2	3.1	3.0	3.0	2.9	2.7	2.6	2.5	2.3
25	4.9	4.5	4.3	4.1	3.9	3.8	3.7	3.6	3.5	3.4	3.3	3.2	3.1	3.0	2.9	2.8	2.6	2.4
30	5.1	4.7	4.4	4.2	4.1	3.9	3.8	3.7	3.6	3.5	3.4	3.3	3.2	3.1	3.0	2.9	2.7	2.5
35	5.3	4.9	4.5	4.4	4.2	4.1	4.0	3.8	3.7	3.6	3.5	3.4	3.3	3.2	3.1	3.0	2.8	2.6
40	5.5	5.0	4.7	4.5	4.4	4.2	4.1	4.0	3.9	3.7	3.6	3.5	3.4	3.3	3.2	3.0	2.9	2.7
45	5.7	5.2	4.9	4.7	4.5	4.4	4.2	4.1	4.0	3.9	3.8	3.6	3.5	3.4	3.3	3.1	3.0	2.8
50	5.9	5.4	5.0	4.9	4.7	4.5	4.4	4.2	4.1	4.0	3.9	3.7	3.6	3.5	3.4	3.2	3.1	2.9
55	6.0	5.5	5.2	5.0	4.8	4.6	4.5	4.4	4.2	4.1	4.0	3.9	3.7	3.6	3.5	3.3	3.2	2.9
60	6.2	5.7	5.4	5.1	5.0	4.8	4.6	4.5	4.4	4.3	4.1	4.0	3.9	3.7	3.6	3.5	3.3	3.0
65	6.5	5.9	5.5	5.3	5.1	5.0	4.8	4.6	4.5	4.4	4.3	4.1	4.0	3.8	3.7	3.6	3.4	3.1
70	6.7	6.0	5.8	5.5	5.3	5.1	5.0	4.8	4.7	4.5	4.4	4.3	4.2	4.0	3.9	3.7	3.5	3.2
75	6.9	6.3	6.0	5.7	5.5	5.3	5.1	5.0	4.9	4.7	4.6	4.5	4.3	4.2	4.0	3.8	3.6	3.4
80	7.3	6.6	6.2	6.0	5.8	5.6	5.4	5.2	5.1	4.9	4.8	4.6	4.5	4.3	4.2	4.0	3.8	3.5
85	7.6	7.0	6.6	6.3	6.0	5.9	5.7	5.5	5.4	5.2	5.0	4.9	4.8	4.5	4.4	4.2	4.0	3.7
90	8.2	7.5	7.1	6.8	6.5	6.3	6.1	5.9	5.8	5.6	5.4	5.3	5.1	4.9	4.8	4.5	4.3	4.0

A small correction value, not over 9 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with 1.5 units of complement, divide by 1.5 or on the basis of control with two units, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 44,

Tabulation of ratios or titers of reactions with present antigen and six units of complement in the test for gonococcus infection

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	5.2	4.9	4.1	4.5	4.3	4.2	4.1	4.0	3.9	3.8	3.7	3.6	3.5	3.4	3.3	3.1	3.0	2.8
10	5.9	5.4	5.2	5.0	4.9	4.7	4.6	4.5	4.4	4.2	4.1	4.0	3.9	3.8	3.6	3.5	3.3	3.1
15	6.3	5.9	5.6	5.4	5.2	5.1	4.9	4.8	4.7	4.6	4.4	4.3	4.2	4.1	3.9	3.8	3.6	3.3
20	6.6	6.1	5.9	5.7	5.5	5.3	5.2	5.1	5.0	4.8	4.7	4.5	4.4	4.3	4.1	4.0	3.8	3.5
25	6.9	6.5	6.1	5.9	5.8	5.6	5.4	5.3	5.2	5.0	4.9	4.8	4.6	4.5	4.3	4.1	4.0	3.7
30	7.2	6.7	6.3	6.2	6.0	5.8	5.7	5.5	5.4	5.2	5.1	4.9	4.8	4.6	4.5	4.3	4.1	3.8
35	7.5	7.0	6.6	6.4	6.2	6.0	5.9	5.7	5.5	5.4	5.3	5.1	5.0	4.8	4.6	4.4	4.2	3.9
40	7.7	7.2	6.8	6.6	6.4	6.2	6.0	5.9	5.7	5.6	5.4	5.3	5.1	5.0	4.8	4.6	4.4	4.1
45	7.9	7.4	7.0	6.8	6.7	6.4	6.3	6.1	5.9	5.8	5.6	5.5	5.3	5.1	5.0	4.8	4.5	4.2
50	8.2	7.7	7.3	7.1	6.8	6.7	6.5	6.3	6.1	6.0	5.8	5.7	5.5	5.3	5.1	4.9	4.7	4.3
55	8.4	7.9	7.5	7.3	7.0	6.8	6.7	6.5	6.3	6.1	5.9	5.8	5.7	5.4	5.2	5.0	4.8	4.5
60	8.7	8.1	7.8	7.5	7.3	7.1	6.8	6.7	6.5	6.3	6.2	6.0	5.8	5.6	5.4	5.2	5.0	4.6
65	9.0	8.4	8.0	7.8	7.5	7.3	7.1	6.9	6.8	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.1	4.8
70	9.4	8.7	8.3	8.0	7.8	7.6	7.4	7.2	7.0	6.8	6.6	6.5	6.2	6.0	5.8	5.6	5.3	4.9
75	9.7	9.0	8.7	8.3	8.1	7.8	7.6	7.5	7.3	7.1	6.9	6.7	6.5	6.2	6.0	5.8	5.5	5.1
80	10.2	9.5	9.0	8.7	8.4	8.2	8.0	7.8	7.6	7.4	7.2	7.0	6.8	6.5	6.3	6.0	5.8	5.4
85	10.8	10.0	9.5	9.2	8.9	8.6	8.4	8.2	8.0	7.8	7.6	7.4	7.2	6.9	6.7	6.4	6.0	5.7
90	11.5	10.8	10.2	9.8	9.5	9.3	9.0	8.8	8.6	8.3	8.1	7.9	7.7	7.5	7.2	6.9	6.5	6.1

A small correction value, not over 9 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with 1.5 units of complement, divide by 1.5 or on the basis of control with two units, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 45

Tabulation of ratios or titers of reactions with present antigen and eight units of complement in the test for gonococcus infection

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	6.6	6.2	5.9	5.8	5.6	5.5	5.4	5.3	5.2	5.0	4.9	4.8	4.7	4.5	4.4	4.2	4.1	3.8
10	7.4	6.9	6.7	6.5	6.3	6.2	6.0	5.9	5.8	5.6	5.5	5.4	5.3	5.1	4.9	4.8	4.5	4.2
15	7.8	7.4	7.2	7.0	6.8	6.6	6.5	6.4	6.2	6.0	5.9	5.8	5.7	5.5	5.3	5.1	4.9	4.5
20	8.3	7.8	7.6	7.4	7.2	7.0	6.8	6.7	6.5	6.4	6.2	6.1	5.9	5.8	5.6	5.4	5.1	4.8
25	8.7	8.2	7.9	7.7	7.5	7.3	7.2	7.0	6.8	6.7	6.5	6.4	6.2	6.0	5.9	5.7	5.4	5.0
30	9.0	8.5	8.1	7.9	7.8	7.6	7.4	7.3	7.1	6.9	6.8	6.6	6.4	6.2	6.1	5.8	5.6	5.2
35	9.4	8.8	8.5	8.2	8.0	7.8	7.7	7.5	7.4	7.2	7.0	6.9	6.7	6.5	6.3	6.0	5.8	5.4
40	9.7	9.1	8.8	8.5	8.3	8.1	7.9	7.8	7.6	7.5	7.3	7.1	6.9	6.7	6.5	6.2	6.0	5.6
45	10.0	9.4	9.0	8.8	8.6	8.4	8.2	8.0	7.9	7.7	7.5	7.3	7.1	6.9	6.7	6.5	6.2	5.8
50	10.4	9.8	9.3	9.1	8.9	8.7	8.5	8.3	8.1	8.0	7.8	7.6	7.4	7.2	7.0	6.7	6.4	6.0
55	10.6	10.0	9.6	9.3	9.1	8.9	8.7	8.5	8.4	8.2	8.0	7.8	7.6	7.4	7.1	6.8	6.5	6.1
60	11.0	10.3	10.0	9.7	9.5	9.2	9.0	8.8	8.6	8.4	8.2	8.0	7.8	7.6	7.4	7.1	6.8	6.3
65	11.4	10.7	10.3	10.0	9.8	9.6	9.3	9.1	9.0	8.7	8.5	8.3	8.1	7.8	7.6	7.3	7.0	6.5
70	11.8	11.1	10.8	10.3	10.1	9.9	9.7	9.4	9.3	9.0	8.8	8.7	8.4	8.2	7.8	7.6	7.3	6.8
75	12.2	11.5	11.2	10.8	10.6	10.3	10.0	9.8	9.6	9.4	9.2	9.0	8.8	8.5	8.2	7.8	7.6	7.1
80	12.8	12.0	11.6	11.3	11.0	10.8	10.6	10.3	10.1	9.8	9.6	9.3	9.1	8.8	8.6	8.3	7.8	7.4
85	13.5	12.7	12.2	11.8	11.7	11.4	11.1	10.9	10.7	10.4	10.1	9.9	9.6	9.3	9.0	8.7	8.3	7.8
90	14.5	13.7	13.1	12.7	12.4	12.1	11.9	11.6	11.4	11.2	10.9	10.7	10.4	10.0	9.7	9.3	8.9	8.4

A small correction value, not over 9 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with 1.5 units of complement, divide by 1.5 or on the basis of control with two units, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

TABLE 46

Tabulation of ratios or titers of reactions with present antigen and twelve units of complement in the test for gonococcus infection

SERUM CONTROL	TEST																	
Per cent of hemolysis	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
5	9.0	8.7	8.4	8.3	8.1	8.0	7.9	7.8	7.7	7.6	7.5	7.3	7.2	7.0	6.9	6.7	6.4	6.1
10	10.1	9.7	9.4	9.3	9.1	9.0	8.9	8.7	8.6	8.5	8.3	8.2	8.0	7.8	7.7	7.5	7.2	6.8
15	10.9	10.4	10.2	9.9	9.8	9.6	9.5	9.4	9.3	9.1	9.0	8.8	8.7	8.5	8.3	8.0	7.8	7.4
20	11.5	11.0	10.8	10.5	10.3	10.2	10.0	9.8	9.7	9.6	9.4	9.3	9.1	8.9	8.7	8.5	8.1	7.7
25	12.0	11.5	11.2	11.0	10.8	10.7	10.5	10.3	10.2	10.0	9.9	9.7	9.5	9.3	9.1	8.8	8.5	8.0
30	12.4	11.9	11.6	11.4	11.2	11.0	10.9	10.8	10.6	10.4	10.2	10.1	9.8	9.7	9.4	9.2	8.8	8.3
35	12.9	12.4	12.0	11.8	11.7	11.4	11.3	11.1	11.0	10.8	10.6	10.4	10.2	10.0	9.8	9.5	9.2	8.7
40	13.3	12.8	12.4	12.2	12.0	11.8	11.6	11.4	11.3	11.2	11.0	10.8	10.6	10.4	10.1	9.8	9.4	8.9
45	13.8	13.2	12.8	12.6	12.4	12.2	12.0	11.8	11.7	11.5	11.3	11.2	10.9	10.8	10.5	10.2	9.8	9.3
50	14.3	13.7	13.3	13.0	12.8	12.6	12.4	12.2	12.1	12.0	11.8	11.5	11.3	11.1	10.8	10.5	10.1	9.6
55	14.7	14.0	13.7	13.5	13.2	13.0	12.8	12.6	12.4	12.2	12.0	11.8	11.6	11.3	11.1	10.8	10.4	9.8
60	15.2	14.5	14.2	13.9	13.7	13.5	13.2	13.1	12.9	12.7	12.4	12.2	12.0	11.8	11.5	11.2	10.8	10.2
65	15.7	15.0	14.7	14.4	14.1	13.9	13.7	13.5	13.2	13.0	12.8	12.6	12.3	12.1	11.9	11.6	11.2	10.6
70	16.3	15.6	15.2	14.9	14.7	14.5	14.2	13.9	13.8	13.6	13.4	13.2	12.9	12.6	12.4	12.0	11.6	11.0
75	16.9	16.2	15.8	15.5	15.2	15.0	14.8	14.5	14.3	14.1	13.9	13.7	13.3	13.0	12.8	12.4	12.0	11.4
80	17.7	16.9	16.6	16.2	15.9	15.7	15.4	15.2	14.9	14.8	14.5	14.2	14.0	13.7	13.4	13.0	12.5	11.8
85	18.7	17.8	17.4	17.0	16.8	16.6	16.3	16.0	15.8	15.6	15.3	15.0	14.8	14.4	14.1	13.7	13.2	12.5
90	20.1	19.1	18.7	18.3	18.0	17.8	17.6	17.3	17.0	16.7	16.5	16.2	15.9	15.6	15.2	14.8	14.2	13.5

A small correction value, not over 9 per cent, has been introduced to allow for the gradual changes in $1/n$ observed in titration curves with increase in K, the amount of complement required for 50-per-cent hemolysis (44).

To determine the value of a test on the basis of the serum control with 1.5 units of complement, divide by 1.5 or on the basis of control with two units, divide by 2 the value of the reaction given in the ratio chart which was calculated on the basis of a similar reaction in the control with one unit of complement.

tion of the tests from tables 35–46 which give the ratios of reactions occurring with the different amounts of complement and the antigens at present in use in the complement-fixation tests for syphilis, tuberculosis, and gonococcus infection, respectively, over ranges of hemolysis in the tests from 5 to 90 per cent by 5-per-cent intervals. Rapid graphic methods have been developed for estimation of the corrected factors and construction of ratio tables (44).

Titers of 1.9 or less are reported "no reaction." Titers of 2 through 2.9 are reported as 2; titers of 3.0 through 3.9 as 3, and so on up to 10. Higher titers are reported as "greater than 10" unless a determination of the end-point titer is to be undertaken.

DETERMINATION OF TITERS GREATER THAN 10

The titer of a serum in the quantitative test is always expressed in terms of the maximum reaction obtainable with 0.05 ml. of the serum. It is recorded in 2 significant figures. In order to determine the end-point titer of highly reactive sera from patients, or from immunized animals, smaller amounts of serum are tested. Under these conditions, the titer of 0.05 ml. of serum is computed from data that determine the linear relationship between complement and serum. In titrating syphilitic serum either of two methods may be used. Method 1 is based upon the linear relationship found between complement and serum when the latter is tested with maximally reactive amounts of antigen. Method 2 is a simplified procedure derived from experience with the more complete titration. The latter, Method 2, has not been used with tubercle or gonococcus antigens but should prove similarly applicable.

In order to select the amounts of reacting serum to be titrated by either method it is necessary to determine by a preliminary test the approximate range of reactivity of the serum.

Preliminary Titer-Range Test

Perform the test as shown in table 47. Record the percentage of hemolysis in each tube. Evaluate the reactions of each quantity of serum in terms of the units of complement required for 50-per-cent hemolysis by reference to table 28 (p. 410).

The result of a typical titer-range test is shown in table 48. From the reactions obtained with the different amounts of serum, estimate the amounts that would give partial reaction with 3, 6, 9, and 12 units of complement as used in Method 1, or with 6 and 9 units of complement in Method 2. For example, in the test recorded in table 48 the reaction with 0.004 ml. of serum was 6.7 and 0.002 ml. of serum did not react. It can be assumed, because of the known proportionality of reactions, that 0.002

TABLE 47

Preliminary titer-range test

TUBE NO.	REACTING SERUM*		NORMAL SERUM†	DOSE OF ANTIGEN OPTIMAL FOR USE WITH 6 UNITS OF COMPLEMENT	DILUTION OF COMPLEMENT CONTAINING 6 UNITS IN 0.1 ML.	SALT SOLUTION	FIXATION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
	Dilution	Amount							
1	1:5	0.05		0.1	0.1	0.05	Standard for the test, see p. 372–374	0.2	Standard for the test, see p. 372–374
2		0.03	0.02	0.1	0.1	0.05		0.2	
3		0.02	0.03	0.1	0.1	0.05		0.2	
4	1:25	0.05		0.1	0.1	0.05		0.2	
5		0.03	0.02	0.1	0.1	0.05		0.2	
6		0.02	0.03	0.1	0.1	0.05		0.2	

Sera having titers in the range of 10 to 20 usually will not react in this test. Hence, when the titer in the test with undiluted serum is greater than 10 and complete hemolysis occurs in the titer-range test, the serum must be retested using 0.05 ml. each of dilutions to 1:2.5, 1:2.78, 1:3.13, 1:3.57, and 1:4.17 with antigen and 6 units of complement and 0.05 ml. each of dilutions to 1:1.67, 1:1.85, 1:2, 1:2.5, and 1:2.78 with antigen and 9 units of complement. If complete hemolysis occurs in this test the titer found in the test of undiluted serum may be reported. Similarly when sera of exceptionally high titer are encountered and no hemolysis occurs with the smallest amounts of serum used in the titer-range test, the serum must be retested using 0.05 ml. each of dilutions to 1:125, 1:143, 1:167, 1:200, 1:227 with antigen and 6 units of complement and 0.05 ml. each of dilutions to 1:83.3, 1:100, 1:111, 1:125, and 1:143 with antigen and 9 units of complement.

* In the titer-range test of cerebrospinal fluids, use 0.1, 0.05, and 0.02 ml. of undiluted fluid and corresponding amounts of a dilution to 1:10 in salt solution. Add salt solution in amounts to bring the total volume in each tube, before fixation, to 0.4 ml.

† Omit normal serum when reacting serum is diluted in salt solution rather than in serum, e.g., in titrations with tubercle or gonococcus antigens and increase correspondingly the amounts of salt solution to be added to bring the total volume before fixation to 0.3 ml.

TABLE 48

Typical result of titer-range test

SYPHILITIC SERUM			% HEMOLYSIS	NUMBER OF UNITS OF COMPLEMENT REQUIRED FOR 50% HEMOLYSIS AS READ FROM TABLE 28
Dilution	Amount pipetted	Equivalent amount	6 units of complement Cardiolipin #72, 1:67	
1:5	0.05	0.01	0	
	0.03	0.006	0	>9.1
	0.02	0.004	35	6.7
1:25	0.05	0.002	100	
	0.03	0.0012	100	
	0.02	0.0008	100	

ml. of serum, tested with 3 units of complement and the corresponding dose of antigen, would give a reaction approximately one-half as great as 0.004 ml. of serum gave in the test with 6 units. Hence, for use in Method 1, 0.05 ml. of a dilution to 1:25, or 0.002 ml. of serum, would be taken as the smallest amount to be tested and a series of dilutions of the serum ranging from 1:5 to 1:25, as given in the footnote of table 29 would be used.

Similarly, for use with 6 units of complement in Method 2 amounts of serum smaller than 0.006 with which no hemolysis occurred and larger than 0.002 with which there was complete hemolysis in the titer-range test, would be used, namely, 0.005, 0.0045, 0.004, 0.0035, and 0.003 ml. Dilutions to contain these amounts in 0.05 ml. would be prepared, namely, 1:10, 1:11.1, 1:12.5, 1:14.3, and 1:16.7. Again, because of the known proportionality of reactions, amounts of serum approximately 1.5 times as great as those used with 6 units would be tested with 9 units, namely, amounts of 0.008, 0.007, 0.006, 0.005, and 0.0045 ml. which would be contained in 0.05 ml. of dilutions to 1:6.25, 1:7.14, 1:8.33, 1:10, and 1:11.1 respectively.

Each dilution used in determining titers over 10 is prepared from the original serum.

Method 1

The method of testing corresponds with that outlined in table 29 (p. 413). Antigen is used in the maximally reactive dose with four amounts of complement—3, 6, 9, and 12 units—and the quantity of serum is varied so that the maximum reaction of each amount, with antigen, is represented by a degree of partial hemolysis with one of the four quantities of complement tested. Include each day the usual controls of complement, antigen, and sensitized cells and a test of the pooled nonreacting serum that is used as diluent of the reacting serum. See p. 422.

Evaluate all reactions in terms of the units of complement required for 50-per-cent hemolysis, by reference to table 28 which gives these values for the different degrees of reaction that may occur with each of the quantities of complement used. See p. 410.

Table 49 illustrates the type of result that is obtained.

Plot the maximum reaction obtained with each amount of serum against the quantities of serum tested. If maximum reactions have been obtained with the different quantities of serum, the points plotted will fall approximately in a straight line. If maximum reactions have not been obtained, repeat the test using appropriate amounts of serum.

The titer is calculated by use of the formula (6)

$$\frac{D[(\mathrm{IS} + \mathrm{A}) - C'] + C'}{\mathrm{IS}}$$

TABLE 49

Results of a typical test to determine titer greater than 10—Method 1

SYPHILITIC SERUM		SERUM CONTROLS			TESTS WITH MAXIMALLY REACTIVE DOSE OF ANTIGEN								MAXIMUM NO. OF UNITS OF COMPLEMENT REQUIRED FOR 50% HEMOLYSIS AS READ FROM TABLE 28	
Dilution in pooled nonreacting serum 0.05 ml.	Equivalent ml. of serum				CARDIOLIPIN ANTIGEN #72,* DILUTIONS IN SALINE									
					1:266	1:133	1:90	1:67	1:67	1:45	1:45	1:33		
		Percentage hemolysis observed with units of complement												
		1	2	3	3		6		9		12		Serum alone (IS)	Serum + antigen (IS + A)
Undiluted	0.05	35	100	100									1.12	
1:5	0.010								0	0	*5*	10		15.0
1:6.25	0.008						0	0	5	5	*35*	50		12.7
1:8.33	0.006						0	0	*40*	50	95	100		9.5
1:12.5	0.004				10	0	*25*	30	100	100				7.2
1:25	0.002				*15*	30	100	100						4.1

* In the test of syphilitic serum to determine titers under 10, cardiolipin antigen No. 72 is diluted to 1:133, 1:67, and 1:33 for use with 3, 6, and 12 units of complement respectively, because these dilutions elicit maximum reactions in the majority of cases with sera having titers within the range up to 10. In determining titers above 10, the use of an additional dose of complement, 9 units, and of 2 doses of antigen with each dose of complement facilitates the determination of the maximum reaction with each quantity of serum tested.

The percentages of hemolysis representing the maximum serum reactions are *italicized*.

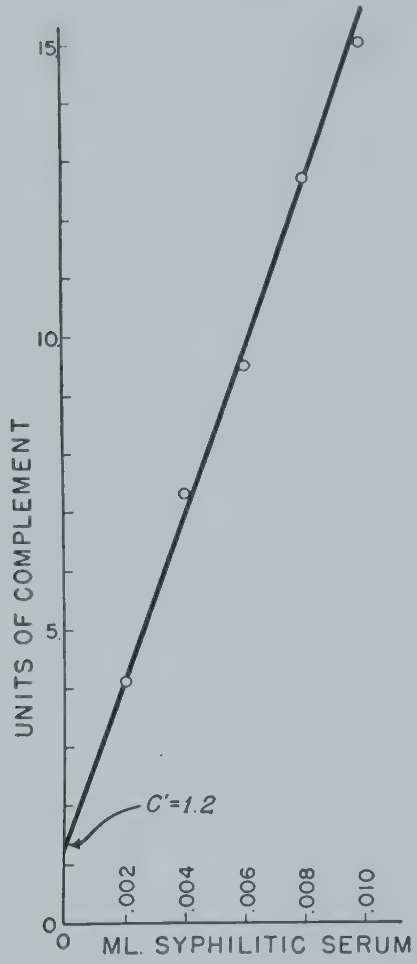


FIG. 51. DETERMINATION OF C' FROM THE LINEAR RELATIONSHIPS OBSERVED BETWEEN COMPLEMENT AND SERUM WITH THE MAXIMALLY REACTIVE DOSE OF ANTIGEN

TABLE 50

Calculation of titer

MAXIMUM REACTION		TITER OF SERUM CALCULATED FROM THE FORMULA $\frac{D[(\text{IS} + \text{A}) - C'] + C'}{(\text{IS})}$
With undiluted serum (IS)	1.12	
With diluted serum + antigen (IS + A)		
Relative concentration of serum 1/*D*		
1:12.5	6.90*	$\frac{12.5\ (6.9-1.2) + 1.2}{1.12} = 65$
1:25	4.05*	$\frac{25\ (4.05-1.2) + 1.2}{1.12} = 65$

* Values taken from fitted straight line.

in which (IS + A) represents the units of complement required for 50-per-cent hemolysis in the presence of diluted serum and the maximally reactive dose of antigen; (IS), the units of complement required for 50-per-cent hemolysis in the presence of undiluted serum; D, the relative serum dilution, *i.e.*, 0.05 ml. divided by the milliliters of serum used in the tests with antigen; and C', the value obtained as shown in figure 51, by extending the straight line representing the linear relationship between serum and complement until it intersects the complement axis, the intercept value being C'.

To determine the titer, substitute in the formula the values of the maximum reaction with any dilution of serum as indicated at any point on the straight line, together with the values determined for (IS) and C', and check the computation by similarly substituting in the formula the reaction of a second quantity of serum. If correctly computed, titers based on the two amounts of serum will agree closely. See "Calculation of Titer," table 50.

Method 2

The method of testing corresponds to that outlined in table 51. The range of quantities to be tested in each case is determined from the titer-range test, as described on p. 438–440. The progression in amounts tested within this range is indicated in the headings of tables 52 and 53 for tests of serum and of tables 54 and 55 for tests of cerebrospinal fluids.

Include each day the usual controls of complement, antigen, and sensitized cells and a test of the pooled nonreacting serum that is used as diluent of the reacting serum (see p. 422).

Record the degree of hemolysis in each tube of the test. Evaluate the reaction of each amount of serum or cerebrospinal fluid by reference to tables 52–55 which give the values D(IS + A) corresponding to the different degrees of hemolysis from 5 to 90 per cent that may be observed in the test with the various amounts of test specimen in the presence of antigen and 6 and 9 units of complement respectively.

The values in the charts were obtained by multiplying the units of complement required for 50-per-cent hemolysis in the presence of diluted specimen and the maximally reactive dose of antigen by the relative dilution.

If the percentage discrepancy—difference divided by the mean—between the maximum reactions obtained respectively with 6 and 9 units exceeds 25 per cent, repeat the test. Otherwise determine the titer by averaging the two maxima and dividing by the value (IS) of the serum control.[9]

[9] Further experience with Method 2 since this manuscript went to press has indicated that the use of two amounts of complement in the titration, 6 and 9 units, contributed to the accuracy of the result in such a small percentage of cases—about 1 per cent—that the test with 9 units is now omitted.

The results of typical tests of serum and cerebrospinal fluid are given in tables 56 and 57.

INTERPRETATION OF RESULTS

The titer that is reported represents the degree of reaction obtained in the complement-fixation test. Slight variations in the titers are of no significance, since they may be found in the examination of duplicate or confirmatory specimens (see footnote 7, p. 409). In the majority of instances however an actual change in reaction is indicated when the percentage discrepancy, $\frac{\text{difference}}{\text{mean}}$, between titers of successive specimens as determined to two significant figures exceeds 29 per cent.

TABLE 51

Determination of titers greater than 10—Method 2

AMOUNT PIPETTED	SERUM* Dilution for use with complement		CARDIOLIPIN #72 DILUTED TO		COMPLEMENT UNITS					SALT SOLUTION	FIXATION	SENSITIZED CELLS	PERIOD FOR HEMOLYSIS
	6 units	9 units	1:67	1:45	1	2	3	6	9				
0.05	Undil.				0.1					0.15	4 hours 3°-6°C.	0.2	15 minutes 37°C.
						0.1				0.15		0.2	
							0.1			0.15		0.2	
0.05	1st		0.1					0.1		0.05		0.2	
0.05	2d		0.1					0.1		0.05		0.2	
0.05	3d		0.1					0.1		0.05		0.2	
0.05	4th		0.1					0.1		0.05		0.2	
0.05	5th		0.1					0.1		0.05		0.2	
0.05		1st		0.1					0.1	0.05		0.2	
0.05		2d		0.1					0.1	0.05		0.2	
0.05		3d		0.1					0.1	0.05		0.2	
0.05		4th		0.1					0.1	0.05		0.2	
0.05		5th		0.1					0.1	0.05		0.2	

* In the test of cerebrospinal fluids use 0.2 ml., undiluted, in the controls and varying amounts from 0.02 to 0.2 ml., undiluted or diluted, as required, in the test with antigen and 6 and 9 units of complement. Before fixation balance with salt solution to a volume of 0.4 ml. in each tube. For typical results see table 57.

The serology of syphilis has provided one of the most valuable laboratory aids in diagnosis that have thus far been devised. The findings have become so useful that in some instances the results of these tests are used as a diagnosis rather than as one of the criteria on which the diagnosis is based.

Several weeks may elapse after exposure to syphilitic infection before the blood of the patient reacts in serologic tests. During this period, sometimes called the incubation stage, *Treponema pallidum*, the inciting microorganism, may be present in the blood, as has been demonstrated by infections resulting from transfusions before the initial lesion developed in the donor. Even after the chancre has appeared, a reaction may not occur for a week or more, and then at first only in very slight degree. Hence, for an early diagnosis, the demonstration of *Trep. pallidum* in fluid from the lesion or enlarged regional lymph nodes is the procedure of choice.

TABLE 52

Values for D(IS + A) *in tests with antigen, 6 units of complement, and serum diluted to 1 in*

% HEMOLYSIS	2.50	2.78	3.13	3.57	4.17	5.00	5.56	6.25	7.14	8.33	10.0	11.1	12.5	14.3	16.7	20.0	22.7
	Equivalent ml. of serum																
	0.020	0.018	0.016	0.014	0.012	0.01	0.009	0.008	0.007	0.006	0.005	0.0045	0.004	0.0035	0.003	0.0025	0.0022
5	23	25	28	32	38	46	51	57	65	76	91	101	114	130	152	182	207
10	21	23	26	30	35	42	46	52	59	69	83	92	104	119	139	166	188
15	20	22	24	28	33	39	43	49	56	65	78	87	98	112	130	156	177
20	19	21	23	26	31	37	41	46	53	62	74	82	93	106	124	148	168
25	18	20	23	26	30	36	40	45	51	60	72	80	90	103	120	144	163
30	17	19	21	24	28	34	38	43	49	57	68	75	85	97	114	136	154
35	17	19	21	24	28	34	37	42	48	56	67	74	84	96	112	134	152
40	16	18	20	23	27	32	36	40	46	53	64	71	80	92	107	128	145
45	16	17	19	22	26	31	34	39	44	52	62	69	78	89	104	124	141
50	15	17	19	21	25	30	33	38	43	50	60	67	75	86	100	120	136
55	15	16	18	21	24	29	32	36	41	48	58	64	73	83	97	116	132
60	14	16	18	20	23	28	31	35	40	47	56	62	70	80	94	112	127
65	14	15	17	19	23	27	30	34	39	45	54	60	68	77	90	108	123
70	13	14	16	19	22	26	29	33	37	43	52	58	65	74	87	104	118
75	12	14	15	17	20	25	27	31	35	41	49	54	61	70	82	98	111
80	12	13	15	17	20	24	26	29	34	39	47	52	59	67	78	94	107
85	11	12	14	16	18	22	24	28	31	37	44	49	55	63	73	88	100
90	10	11	13	14	17	20	22	25	29	33	40	44	50	57	67	80	91

When smaller amounts of serum are to be tested a similar progression is used, hence the values in the table merely undergo a 10-fold change.

In the secondary stage the titer of the blood serum is usually high. In the late stage of an unrecognized infection, the titer may vary widely in different individuals. Marked reactions may occur with specimens from patients who show few, if any, clinical signs. On the other hand, blood from a patient with syphilis of the central nervous system may have a low titer. Following treatment, particularly in primary and secondary syphilis, the serologic reactivity of the serum may continue to rise initially, but falls rapidly thereafter, and may disappear in three to six months, when treatment has been successful (45, 45a). As the treated early case is followed, a marked rise in titer precedes a clinical relapse or indicates reinfection. When treatment is started late in the disease, serologic reactivity in the serum may persist for many years.

TABLE 53

Values for D(IS + A) *in tests with antigen, 9 units of complement, and serum diluted to 1 in*

% HEMOLYSIS	1.67	1.85	2.00	2.50	2.78	3.13	3.57	4.17	5.00	5.56	6.25	7.14	8.33	10.0	11.1	12.5	14.3
	Equivalent ml. of serum																
	0.03	0.027	0.025	0.02	0.018	0.016	0.014	0.012	0.01	0.009	0.008	0.007	0.006	0.005	0.0045	0.004	0.0035
5	20	23	24	31	34	38	44	51	61	68	76	87	102	122	135	153	174
10	19	21	23	29	32	36	41	48	58	64	72	82	96	115	128	144	164
15	18	20	22	28	31	34	39	46	55	61	69	79	92	110	122	138	157
20	18	20	21	27	29	33	38	44	53	59	66	76	88	106	118	133	152
25	17	19	20	26	28	32	36	43	51	57	64	73	85	102	113	128	146
30	17	19	20	25	28	31	36	42	50	56	63	71	83	100	111	125	143
35	16	18	19	24	27	30	35	40	49	54	61	69	81	97	108	121	139
40	16	18	19	24	26	30	34	40	48	53	59	68	79	95	105	119	136
45	15	17	18	23	26	29	33	38	46	51	58	66	77	92	102	115	132
50	15	17	18	23	25	28	32	38	45	50	56	64	75	90	100	113	129
55	15	16	18	22	24	28	31	37	44	49	55	63	73	88	98	110	126
60	14	16	17	21	24	27	30	35	43	47	53	61	71	85	94	106	122
65	14	15	16	21	23	26	29	34	41	46	51	59	68	82	91	103	117
70	13	15	16	20	22	25	28	33	40	44	49	56	66	79	88	99	113
75	13	14	15	19	21	24	27	32	38	42	48	54	63	76	84	95	109
80	12	14	15	18	20	23	26	30	37	41	46	52	61	73	81	91	104
85	11	13	14	17	19	21	24	28	34	38	43	49	57	68	75	85	97
90	10	11	12	16	17	19	22	26	31	34	39	44	52	62	69	78	89

When smaller amounts of serum are to be tested a similar progression is used, hence the values in the table merely undergo a 10-fold change.

The tissues of all individuals do not react uniformly. The blood of some will react to the same stimulus much more quickly and to a higher degree than that of others. Such findings are to be expected in all types of immunologic reaction; for example, one horse may produce a much more potent antitoxin than another, even though the same dose of toxin is administered. Similar variability is evident in the titer of the serologic reaction in syphilis.

Low titers are obtained with specimens from patients who have recently acquired the infection, from those who have received a large amount of treatment, from certain late cases of syphilis, and occasionally, from persons who do not have syphilis. Titers reported as greater than 10 represent a very wide degree of reaction, that is, from slightly over 10, such as 11 or 12, to excessively high titers of 300 to 500, and even up to 2,000.

TABLE 54

Values for D(IS + A) *in tests with antigen, 6 units of complement and cerebrospinal fluid in the given amounts*

% HEMOLYSIS	0.1	0.09	0.08	0.07	0.06	0.05	0.045	0.04	0.035	0.03	EITHER 0.025	OR* 0.024	0.022	0.02	EITHER 0.018	OR* 0.0175	EITHER 0.016	OR* 0.015	0.014	0.012
5	18	20	23	26	30	36	40	46	52	61	73	76	83	91	101	104	114	121	130	152
10	17	18	21	24	28	33	37	42	47	55	66	69	76	83	92	95	104	110	119	139
15	16	17	20	22	26	31	35	39	45	52	62	65	71	78	87	89	98	104	112	130
20	15	16	19	21	25	30	33	37	42	49	59	62	67	74	82	84	93	98	106	124
25	14	16	18	21	24	29	32	36	41	48	58	60	66	72	80	82	90	96	103	120
30	14	15	17	19	23	27	30	34	39	45	54	57	62	68	75	78	85	90	97	114
35	13	15	17	19	22	27	30	34	38	45	54	56	61	67	74	76	84	89	96	112
40	13	14	16	18	21	26	28	32	37	43	51	53	58	64	71	73	80	85	92	107
45	12	14	16	18	21	25	28	31	35	41	50	52	56	62	69	71	78	82	89	104
50	12	13	15	17	20	24	27	30	34	40	48	50	55	60	67	68	75	80	86	100
55	12	13	15	17	19	23	26	29	33	39	46	48	53	58	64	66	73	77	83	97
60	11	12	14	16	19	22	25	28	32	37	45	47	51	56	62	64	70	74	80	94
65	11	12	14	15	18	22	24	27	31	36	43	45	49	54	60	62	68	72	77	90
70	10	12	13	15	17	21	23	26	30	35	42	43	47	52	58	59	65	69	74	87
75	10	11	12	14	16	20	22	25	28	33	39	41	45	49	54	56	61	65	70	82
80	9.4	10	12	13	16	19	21	24	27	31	38	39	43	47	52	54	59	63	67	78
85	8.8	10	11	13	15	18	20	22	25	29	35	37	40	44	49	50	55	59	63	73
90	8.0	8.9	10	11	13	16	18	20	23	27	32	33	36	40	44	46	50	53	57	67

When smaller amounts of cerebrospinal fluid are to be tested a similar progression is used; hence the values in the table merely undergo a 10-fold change.

* A choice of amounts is allowed in order to avoid making more than one dilution of cerebrospinal fluid for the test with 6 units of complement.

A relatively small percentage of sera cannot be titrated accurately. In some instances the serum is anticomplementary; that is, it will react with complement alone without the addition of antigen. This anticomplementary quality of serum alone tends to mask the reaction in the presence of antigen so that an accurate expression of the titer cannot be given. However, usually the presence or absence of a reaction may be indicated

TABLE 55

Values for D(IS + A) in tests with antigen, 9 units of complement and cerebrospinal fluid in the given amounts

% HEMOLYSIS	0.15	0.14	0.12	0.11	0.09	0.08	0.07	0.06	0.05	0.045	EITHER 0.04	OR* 0.038	EITHER 0.035	OR* 0.034	0.03	EITHER 0.028	OR* 0.0275	EITHER 0.025	OR* 0.024	0.02	0.018
5	16	17	20	22	27	31	35	41	49	54	61	64	70	72	81	87	89	98	102	122	135
10	15	16	19	21	26	29	33	38	46	51	58	61	66	68	77	82	84	92	96	115	128
15	15	16	18	20	24	28	31	37	44	49	55	58	63	65	73	78	86	88	92	110	122
20	14	15	18	19	24	27	30	35	42	47	53	56	61	62	71	75	77	85	88	106	118
25	14	15	17	19	23	26	29	34	41	45	51	54	58	60	68	72	74	82	85	102	113
30	13	14	17	18	22	25	29	33	40	44	50	53	57	59	67	71	73	80	83	100	111
35	13	14	16	18	22	24	28	32	39	43	49	51	55	57	65	69	71	78	81	97	108
40	13	14	16	17	21	24	27	32	38	42	48	50	54	56	63	67	69	76	79	95	105
45	12	13	15	17	20	23	26	31	37	41	46	48	53	54	61	65	67	74	77	92	102
50	12	13	15	16	20	23	26	30	36	40	45	47	51	53	60	64	65	72	75	90	100
55	12	13	15	16	20	22	25	29	35	39	44	46	50	52	59	62	64	70	73	88	98
60	11	12	14	15	19	21	24	28	34	38	43	45	49	50	57	60	62	68	71	85	94
65	11	12	14	15	18	21	23	27	33	36	41	43	47	48	55	58	60	66	68	82	91
70	11	11	13	14	18	20	23	26	32	35	40	42	45	46	53	56	57	63	66	79	88
75	10	11	13	14	17	19	22	25	30	34	38	40	43	45	51	54	55	61	63	76	84
80	10	10	12	13	16	18	21	24	29	32	37	38	42	43	49	52	53	58	61	73	81
85	9.1	10	11	12	15	17	19	23	27	30	34	36	39	40	45	48	49	54	57	68	75
90	8.3	8.9	10	11	14	16	18	21	25	28	31	33	35	36	41	44	45	50	52	62	69

When smaller amounts of cerebrospinal fluid are to be tested a similar progression is used; hence the values in the table merely undergo a 10-fold change.

* A choice of amounts is allowed in order to avoid making more than one dilution of cerebrospinal fluid for the test with 9 units of complement.

TABLE 56

Typical result of test of serum to determine titer greater than 10, by Method 2

SERUM, 0.05 ML.		PERCENTAGE OF HEMOLYSIS WITH					(IS)	(IS + A)	D(IS + A) READ FROM TABLES 52 AND 53	TITER
		UNITS OF COMPLEMENT								
					6	9				
					Cardiolipin No. 72					
Dilution	Equivalent amount of serum	1	2	3	1:67	1:45				
Undil.	0.05	25	95	100			1.23			
1:10	0.005				10			8.3	83	
1:11.1	0.0045				15			7.8	87	
1:12.5	0.004				25			7.2	90	
1:14.3	0.0035				30			6.8	*97*	$\frac{\frac{97+92}{2}}{1.23} = 77$
1:16.7	0.003				70			5.2	87	
1:6.25	0.008					0		>12.2	>76	
1:7.14	0.007					5		12.2	87	
1:8.33	0.006					20		10.6	88	
1:10	0.005					45		9.2	*92*	
1:11.1	0.0045					70		7.9	88	

but such a result should always be confirmed by examination of additional specimens. In other cases an atypical reaction may be obtained which varies in titer in the several tubes of the test. In figure 52 this type of reaction is compared with the usual typical reaction. Atypical reaction may occur in nonsyphilitic individuals as well as in those who have syphilis. In any instance where the specimen has proved to be unsatisfactory for examination or the findings are atypical, another specimen should be submitted.

TABLE 57

Typical result of test of cerebrospinal fluid to determine titer greater than 10, by Method 2

CEREBROSPINAL FLUID		PERCENTAGE OF HEMOLYSIS WITH UNITS OF COMPLEMENT					(IS)	(IS + A)	*D*(IS + A) READ FROM TABLES 54 AND 55	TITER
Dilution and Amount Pipetted	Equivalent Amount	1	2	3	6	9				
					Cardiolipin No. 72					
					1:90	1:45				
Undil. 0.2	0.200	45	100	100			1.04			
1:5, 0.11	0.022				10			8.3	76	
1:5, 0.10	0.020				15			7.8	*78*	$\dfrac{\dfrac{78 + 71}{2}}{1.04} = 72$
1:5, 0.09	0.018				35			6.7	74	
1:5, 0.08	0.016				80			4.7	59	
1:5, 0.07	0.014				100					
1:5, 0.17	0.034					10		11.5	68	
1:5, 0.15	0.030					20		10.6	*71*	
1:5, 0.14	0.028					35		9.7	69	
1:5, 0.12	0.024					85		6.8	57	
1:5, 0.10	0.020					100				

The interpretation of a reaction should be particularly guarded in such conditions as acute upper respiratory infection, following vaccination against smallpox or typhoid fever, after some antitoxins, and during and immediately after febrile diseases generally, and particularly after malaria. Further specimens should be examined and antisyphilitic treatment withheld. When these successive specimens reveal a falling titer, observation should be continued for a three-month period, during which most of these reactions will disappear. It might be mentioned that with the technic and antigens herein described, reactions of a significant degree in these conditions occur much less frequently than reports in the literature based on certain of the tests in common use would indicate (46–52). The heterophile antibody which is associated with infectious mononucleosis has not interfered with the results of the test in our experience.

The evaluation of the findings in the complement-fixation test for syphilis is dependent on the degree of reaction obtained. A titer which is greater than 10 rarely occurs with specimens from patients who do not have syphilis, except in the case of those with yaws or some other disease incited by spirochetes.

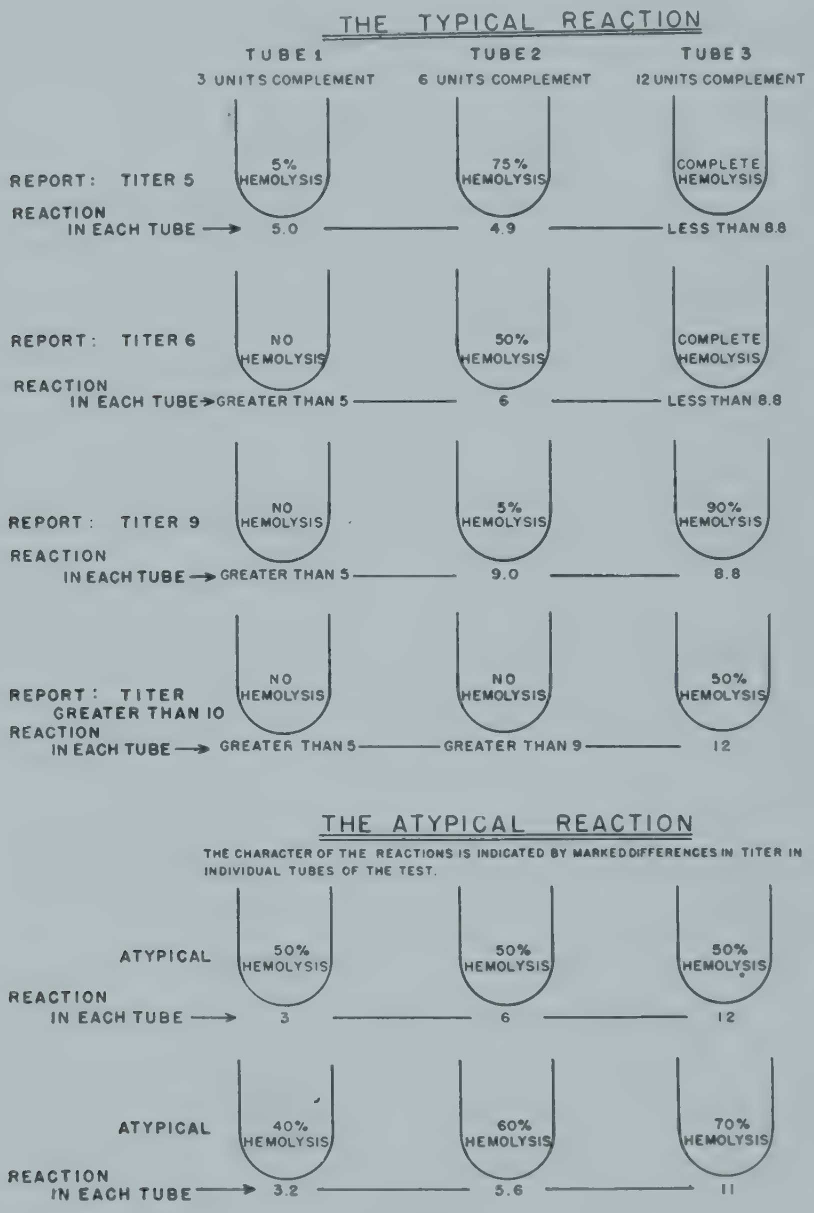

THE TYPICAL REACTION
TUBE 1
3 UNITS COMPLEMENT
TUBE 2
6 UNITS COMPLEMENT
TUBE 3
12 UNITS COMPLEMENT
REPORT: TITER 5
5% HEMOLYSIS
75% HEMOLYSIS
COMPLETE HEMOLYSIS
REACTION IN EACH TUBE
5.0
4.9
LESS THAN 8.8
REPORT: TITER 6
NO HEMOLYSIS
50% HEMOLYSIS
COMPLETE HEMOLYSIS
REACTION IN EACH TUBE
GREATER THAN 5
6
LESS THAN 8.8
REPORT: TITER 9
NO HEMOLYSIS
5% HEMOLYSIS
90% HEMOLYSIS
REACTION IN EACH TUBE
GREATER THAN 5
9.0
8.8
REPORT: TITER GREATER THAN 10
NO HEMOLYSIS
NO HEMOLYSIS
50% HEMOLYSIS
REACTION IN EACH TUBE
GREATER THAN 5
GREATER THAN 9
12
THE ATYPICAL REACTION
THE CHARACTER OF THE REACTIONS IS INDICATED BY MARKED DIFFERENCES IN TITER IN INDIVIDUAL TUBES OF THE TEST.
ATYPICAL
50% HEMOLYSIS
50% HEMOLYSIS
50% HEMOLYSIS
REACTION IN EACH TUBE
3
6
12
ATYPICAL
40% HEMOLYSIS
60% HEMOLYSIS
70% HEMOLYSIS
REACTION IN EACH TUBE
3.2
5.6
11

FIG. 52

If early syphilis is suspected, 10 ml. of blood for the complement-fixation test should be submitted at weekly intervals until eight weeks have elapsed following the appearance of the primary lesion, unless evidence of syphilis is obtained earlier. If a reaction is reported and no history or evidence of the disease can be elicited, at least one additional specimen should be examined. In a nonsyphilitic individual or a normal infant born of a syphilitic mother, the reaction may disappear without treatment, usually within a period of three months. The history accompanying specimens of this character should give an explanatory statement, so that special tests can be made if the findings indicate that they may be of value. This procedure is especially to be urged if a relatively low titer is obtained or the character of the reaction is not entirely typical. The tube should be filled nearly full of blood so that adequate serum will be available. Physicians must realize that essential clinical data are just as helpful to the laboratory service as are the results of laboratory examinations to clinicians in the practice of medicine.

If the titer of successive specimens continues low and there are no signs of syphilitic infection, a specimen of cerebrospinal fluid should be examined. An X-ray of the chest may also be desirable to learn if the large blood vessels have been affected.

In tuberculosis, titers up to 400 have been observed in experience thus far. The zone of reaction that requires special precaution in the interpretation of results appears much broader with this bacterial antigen than it is in syphilis. Hence in the evaluation of the findings obtained, titers in the range up to 3.0 may not be of significance in the absence of clinical or other signs of tuberculosis. Titers greater than 3.0 are relatively infrequent when signs of tuberculosis are absent. In the arrested or inactive stages of the disease or in early but slightly active infection, the reaction may be of low degree or absent, whereas, with more active or advanced disease higher titers are usually obtained. Reactions, however, are sometimes not obtained with specimens from patients with advanced or rapidly progressing miliary tuberculosis. The clinician has the safeguards of sputum and X-ray examinations and also the tuberculin test as a basis for the interpretation of the results of the serologic test of the specific activity in the blood serum.

In the test with gonococcus antigens, our practical experience is limited. Titers over 65 have not yet been recorded in tests of human serum. Although the greater proportion of sera from cases of active or recently active gonorrhea have had titers over 2, a significant number from early uncomplicated or sulfonamide-treated cases failed to react in the quantitative complement-fixation test. The absence of a reaction does not exclude the presence of the gonococcus.

None of a group of one hundred and fifty-four specimens from healthy persons with no recorded history of gonorrhea had titers over 2 with the gonococcus cellular antigen. Higher titers were observed with a number of sera from cases of syphilis, arthritis, and pneumonia in which there was no record of gonorrhea. The possibility of previous or latent gonococcus infection could not always be definitely excluded. However, it is evident that the presence of a reaction does not necessarily indicate the presence of gonococcus infection and that infections with certain other species of cocci must be considered in the evaluation of the results of the gonococcus complement-fixation test.

Over the years serologists have met many difficulties and have solved many problems but, as the subtle reactivities of serum with antigen in the presence of other constituents of the tests are revealed, revision and refinement of technical standards must be made. It is remarkable that the serum expressed from the clot of the labile blood plasma as it is transformed through coagulation is as uniform as it is from the standpoint of the serologic tests (59). But the serologist must be alert to detect variation—the exceptions that occur and become strikingly evident the more delicately balanced the serologic test. Reactions, in the serologic tests for syphilis, the diagnostic significance of which is not clear, have been characterized by some syphilologists as "false positive" or "biologic false positive" reactions. These terms are misnomers. A false positive connotes a diagnosis which in the last analysis is the responsibility of the clinician; he should interpret the significance of and evaluate all data. No biologic reaction is false—quite the contrary; it may not be clearly understood by the clinician, but the serologist is familiar with anticomplementary, nonspecific, and cross-reactions that are not uncommon in serologic tests. He also knows that the serum constituents and the dispersion of the antigenic substances are to an extent labile, in particular affected by the diluent, and to a marked degree in some of the serologic tests. All these problems are to be solved with advancing knowledge.

PRELIMINARY TESTS FOR SYPHILIS

The general instructions regarding technical procedures given in the description of the quantitative method of complement fixation apply also to these preliminary tests.

Two tests are made: either two 1-tube complement-fixation tests, with different antigens, or a 1-tube complement-fixation test and a sensitive precipitation test, p. 462.

PRELIMINARY COMPLEMENT-FIXATION TEST

Preparation and Standardization of Reagents

The reagents used in this test are prepared and standardized in the same manner as those used in the quantitative complement-fixation test. The dilution of antigen employed is that which has been found to give maximum reaction with sera of relatively low degrees of activity, i.e., the dilution used in the quantitative test with 3 units of complement.

The Test

To 0.05 ml. of patient's serum, add 0.1 ml. of antigen, 0.1 ml. of the dilution of complement that contains 3 units in this amount, and 0.05 ml. of physiologic salt solution. Allow for fixation two hours in the refrigerator at from 3° to 6°C. followed by one-half hour in a water bath at 37°C. Add 0.2 ml. of sensitized cells and incubate in a water bath for fifteen minutes at 37°C. With the first and last sera tested by this procedure on a given date, include quantitative complement-fixation tests of two known reacting sera of different degrees of reactivity, and of a normal serum. In the case of normal serum, omit the test with 6 and 12 units of complement but add a test with 2 units. Include also controls of the anticomplementary properties of 0.1 ml. of antigen in the presence of 1 and 2 units of complement, as well as of 1 and 2 units of complement alone. Control also the activity of the dilution of complement containing 3 units in 0.1 ml. by including a test of 0.05 ml. of this dilution.

All cerebrospinal fluids receive a complete quantitative complement-fixation test, but as a technical control a 1-tube test of each specimen is performed by a separate group of workers; 0.2 ml. of cerebrospinal fluid is used and the test is the same as the preliminary test of serum except that four hours in the refrigerator at from 3° to 6°C. are allowed for fixation.

Read all tests and controls by comparison with the color standard and record the per cent hemolysis observed.

Perform quantitative tests on the following day with all specimens giving 90-per-cent or less hemolysis.

Specimens that fail to give a significant degree of reaction in either of the preliminary tests may be reported as giving no reaction in serologic tests for syphilis.

SCHEDULE OF PROCEDURES FOR COMPLEMENT-FIXATION TESTS FOR SYPHILIS

Assume that standardized amboceptor and antigen have been secured and that sheep and guinea pigs are available. The following schedule of procedures covering two working days has these objectives:

First day:

1. To prepare required reagents and perform and report the results of preliminary tests of specimens.
2. To prepare in part the complement and sheep cells for use the following day.

Second day:

1. To complete the preparation of required reagents.

2. To perform and report the results of quantitative complement-fixation tests.

The order of procedure may be modified depending upon the staff available and the volume of the work. If the volume of the work necessitates the performance of tests each day, the performance of preliminary tests of one group of specimens and of the quantitative complement-fixation tests with specimens having received preliminary tests the preceding day, may be carried out simultaneously. Opening, numbering, and separation from the clot may be carried out as soon as specimens are received, so that when the time comes to prepare specimens for preliminary tests, the majority will be ready for inactivation. Proper storage of serum is imperative.

FIRST DAY

Bleed sheep (p. 68, 376).

Bleed guinea pigs (p. 83, 381).

During the hour allowed for separation of guinea-pig serum from the clot:

Wash sheep cells (p. 376–377) for use in tests of guinea-pig sera and in preliminary tests of specimens.

Dilute amboceptor and antigen. (Follow directions furnished with each preparation.)

Prepare 5-per-cent cells suspension (p. 377) and hemoglobin solution (p. 374).

Prepare color standards (table 15, p. 375).

Sensitize cells for use in tests of guinea-pig sera (p. 377).

Separate guinea-pig serum from the clot and test for hemolytic activity, natural amboceptor, and nonspecific fixability (p. 382–383).

While tests for nonspecific fixability are incubating, prepare and inactivate specimens for preliminary tests (p. 420–422).

Pool the guinea-pig sera that are satisfactory for use (p. 382, 383).

Perform complement titrations, prepare and check dilutions required for preliminary tests and controls (p. 383–386).

Perform preliminary tests of specimens (p. 452–453, 462).

Wash sheep cells twice for following day's work (p. 376–377).

Stopper specimen tubes and store overnight in the refrigerator.

Report results of preliminary tests (p. 453, 462).

SECOND DAY

Finish washing sheep cells, prepare 5-per-cent suspension (p. 376–377) and hemoglobin solution (p. 374).

Dilute amboceptor and antigen. (Follow directions furnished with each preparation.)

Titrate complement (p. 383–386).

Prepare color standards while complement titration is in the 37°C. water bath. Table 15, p. 375.

Prepare and check complement dilutions for use in tests (p. 386).

Pick out and reinactivate specimens requiring quantitative tests (p. 421, 422, 453).

Perform quantitative complement-fixation tests (p. 422–425).

Report results of tests (p. 425–438).

QUANTITATIVE PRECIPITATION TEST FOR EVIDENCE OF SYPHILIS

GENERAL DIRECTIONS

The quantitative precipitation test for evidence of syphilis is performed when it is considered essential that a report be furnished immediately. In such instances the complement-fixation test is performed subsequently and the final report then made. The test is also performed supplementary to the quantitative complement-fixation test when the results of the latter are unsatisfactory; that is, when the serum is anticomplementary, when the serum reacts but not in a quantitatively characteristic manner, or when the results of different tests disagree or are not in accord with clinical findings. The result of the precipitation test may then be incorporated in a special report.

A preliminary precipitation test (p. 462) is used as a screening procedure with all sera in conjunction with a preliminary complement-fixation test (p. 452).

Glassware

Pipettes.—Use pipettes as follows: for measuring stock solutions of antigen and antigen diluent, 1-ml. graduated in 0.01 ml., 5-, or 10-ml. according to the amount; for measuring serum and antigen suspension for tests, 0.2-ml. pipettes graduated in 0.01 ml.; for adding salt solution to serum in the preparation of dilutions, 1.0-ml. pipettes graduated in 0.01 ml., or 5-ml. pipettes graduated in 0.1 ml. according to the amount; for adding 2 ml. of salt solution to the serum and antigen suspension in the tests, 10-ml. pipettes graduated in 0.1 ml.

Tubes.—For mixing antigen suspensions, use tubes 15- by 100-mm. with spout; for heating antigen suspensions, use round-bottomed vials 15- by 100-mm. fitted with screw caps lined with composition, or aluminum or tin foil; for performing tests, use tubes 11- by 75-mm. Tubes in which antigen suspensions are prepared and those in which tests are performed must be clear.

Beakers.—For mixing antigen suspensions, use low-form beakers with spout, 5-, 10-, 20-, 30-, or 50-ml. according to the amount.

Reagents

Blood Serum.—Prepare in the same manner as for the complement-fixation test (see p. 421). If serum has been removed from the clot for more than twenty-four hours, it may be necessary to centrifuge for ten minutes at 1400 r.p.m. and separate from the sediment before testing.

Cerebrospinal Fluid.—Cerebrospinal fluids are not tested in the precipitation procedure, since the standardization has not been completed.

Cardiolipin-Lecithin-Cholesterol Antigen.—*Preparation of Stock Antigen* (23, 23a). Prepare a stock solution in absolute alcohol (U.S.P.) of 0.03-per-cent cardiolipin, 0.75-per-cent lecithin, and 0.25-per-cent cholesterol (C. P. Eimer and Amend). These amounts and proportions provide an antigen of a conservative degree of sensitivity, little subject to serum prozone and inhibited reactions (53). In these respects this antigen is superior to crude tissue extract antigen (54, 55).

A slightly less concentrated antigen, 0.024 per cent of cardiolipin, 0.6 per cent of lecithin, and 0.2 per cent of cholesterol is optimum for tests with diluted sera.

These antigens have been tentatively adopted for routine use (23, 56). More experience is necessary, however, to determine the optimum sensitivity in relation to both syphilitic and nonsyphilitic reactions.

Preparation of Antigen Suspension.—The antigen suspension used in the tests may be prepared in tubes or in beakers. The suspension is prepared in tubes as a 1:2.05 dilution of the antigen.

Select two tubes of like diameter (15- by 100-mm.), preferably with a spout. Into one, pipette 1 ml. of the stock antigen; into the other, pipette 1.05 ml. of 0.4-per-cent sodium chloride solution. Cork the tubes tightly and place them in an incubator at 37°C. for ten minutes to assure a uniform temperature at the time of mixing. Remove the corks, and, without spilling, rapidly pour the diluent onto the antigen; then mix by pouring from one tube to the other six times. Pour the suspension into a round-bottomed vial (15- by 100-mm.) fitted with a screw cap lined with composition, aluminum, or tin foil. Do not use unprotected cork. Screw on the cap securely and place the vial in a water bath at 56°C. for fifteen minutes. Evaporation must not occur. Leave the suspension in the closed vial at room temperature for at least five minutes.

Before use, mix the contents of the vial thoroughly to take up all condensed moisture and perform control tests of the antigen suspension with physiologic salt solution, a nonreacting serum, and a strongly reacting serum. Do not use an antigen suspension that is unsatisfactory in these controls; that is, one that does not redisperse in salt solution and the nonreacting serum or does not give marked precipitation with the reacting serum. Pool all vials of satisfactory suspensions.

Suspensions are most satisfactorily prepared, however, in beakers as a 1:2.0 dilution of the antigen.

Small low-form beakers with spout are used and the size of the beaker is suited to the amount of the material. Thus for the smallest amount of suspension prepared, use 5-ml. beakers; into one, pipette exactly 1.0 ml. of 0.4-per-cent sodium chloride solution and into another, 1.0 ml. of stock antigen. Pour the salt solution onto the antigen moderately rapidly and then mix by pouring from one beaker to the other six times. Pour into a screw-cap vial, heat, and perform control tests as usual. Use 10-ml. beakers for 2 to 3 ml. of antigen, 20-ml. beakers for 4 to 7 ml., etc. Dispense 2-ml. portions of the suspension into several screw-cap vials for heating. This method of dilution may be used for as much as 10 ml. of stock antigen.

Before use perform control tests of the antigen suspension with physiologic salt solution, a strongly reacting serum, and a nonreacting serum. Do not use an antigen suspension that is unsatisfactory in these controls; that is, one which does not redisperse in salt solution and the nonreacting serum or does not give marked precipitation with the reacting serum.

These antigen suspensions are satisfactory for use for at least six hours when kept at room temperature.

Salt Solutions.—Isotonic salt solution for use in making the final dispersion of the test mixture of serum and antigen (*F69A*).

Physiologic salt solution for diluting serum (*F64*).

0.4-per-cent sodium chloride solution for diluting antigen.

Minimum 2+ Standards for Reading

Minimum 2+ standards for the reading of tests have been developed. They are prepared from a suspension of Hyflo Super-Cel (Johns Manville) to supply the particles, from a suspension of glass particles to give the necessary turbidity, from serum, and from 0.85-per-cent sodium chloride solution. The minimum 2+ standards are prepared as in table 58.

Hyflo Super-Cel Suspension.—Place 2.3 grams of the earth in a 50-ml. graduate and add enough 0.85-per-cent sodium chloride solution to bring the volume to 50 ml. Shake thoroughly and allow to stand for exactly five minutes. Then, with suction draw off the supernatant into another 50-ml. graduate, shake thoroughly and again let stand for five minutes. Repeat the procedure for a total of six times. The sixth supernatant constitutes the stock suspension. Adjust if necessary to match a previously standardized suspension, just saturate with chloroform, and store in a tightly closed container of pyrex glass, in the cold. Before removing a portion bring to room temperature and shake thoroughly.

Check the uniformity of the suspension by turbidity measurements in a Klett-Summerson photoelectric colorimeter. For this determination, make a 1:10 dilution of the suspension in 0.85-per-cent sodium chloride solution. With an accurate "to deliver" pipette place 1 ml. of the suspension in a calibrated 10-ml. flask and make up to volume. About 5 ml. of the dilution is transferred to a standard colorimeter tube and readings made with the zero set for a blank of 0.85-per-cent sodium chloride solution using a blue filter No. 42. Since the suspension tends to settle, care and speed are necessary in all transfers to insure uniform sampling, and each tube is shaken just before it is placed in the instrument. In a limited experience, the readings for 1:10 dilutions of satisfactory suspensions were between 240 and 260. The Super-Cel content of the stock suspensions as determined gravimetrically was between 10.8 and 13.0 mg. per ml. Adjustment can be made by the addition of salt solution or the removal of clear supernatant and the turbidity determination repeated. Check all new suspensions with a previously standardized one.

Pyrex Glass Suspension.—The stock suspension of pyrex glass is prepared essentially as described on page 26. The subsequent standardization is modified somewhat (56): Adjust the original suspension to give a reading of 590 in the Klett-Summerson colorimeter. (The dry solid content of four such suspensions varied from 0.298 to 0.306 per cent.) Next prepare a dilution of approximately 1:4.8 to obtain a suspension which gives a reading of 260 to 280 in the colorimeter. Just saturate with chloroform. This constitutes the stock pyrex glass suspension used in the preparation of the minimum 2+ standards. Check all new suspensions with a previously standardized one. Store in a tightly closed container of pyrex glass in the cold.

TABLE 58

Preparation of minimum 2+ standards

	HYFLO SUPER-CEL SUSPENSION	PYREX GLASS SUSPENSION	SERUM	0.85% NaCl
	ml.	*ml.*	*ml.*	*ml.*
For tests with undiluted serum				
tube 1 with 0.02 ml. antigen	0.025	0.22	0.1	1.8
tube 2 with 0.04 ml. antigen	0.05	0.44	0.1	1.6
tube 3 with 0.06 ml. antigen	0.075	0.66	0.1	1.4
For tests with diluted serum				
with 0.02 ml. stock antigen	0.025	0.22	0.0	1.9
with 0.02 ml. less concentrated antigen	0.025	0.18	0.0	1.9

The standards should be prepared on the day they are used. They must be thoroughly shaken when in use.

THE QUANTITATIVE TEST (56)

In the quantitative precipitation test a single amount of serum, 0.1 ml., is used with three amounts of antigen, 0.02, 0.04, 0.06 ml. It has been demonstrated that these multiple doses of antigen react with proportional amounts of serum to give a similar degree of precipitation. For example, with a serum of slight reactivity 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 ml. of the serum gave a 2+ reaction with 0.01, 0.02, 0.03, 0.04, 0.05, and 0.06 ml. of antigen respectively. Likewise, when a pool of highly reacting serum was diluted with a pool of nonreacting serum the same straight-line relationship held (Figure 52A). By the use of three different proportions of serum and antigen the inhibiting effect of excess of either reagent is detectable.

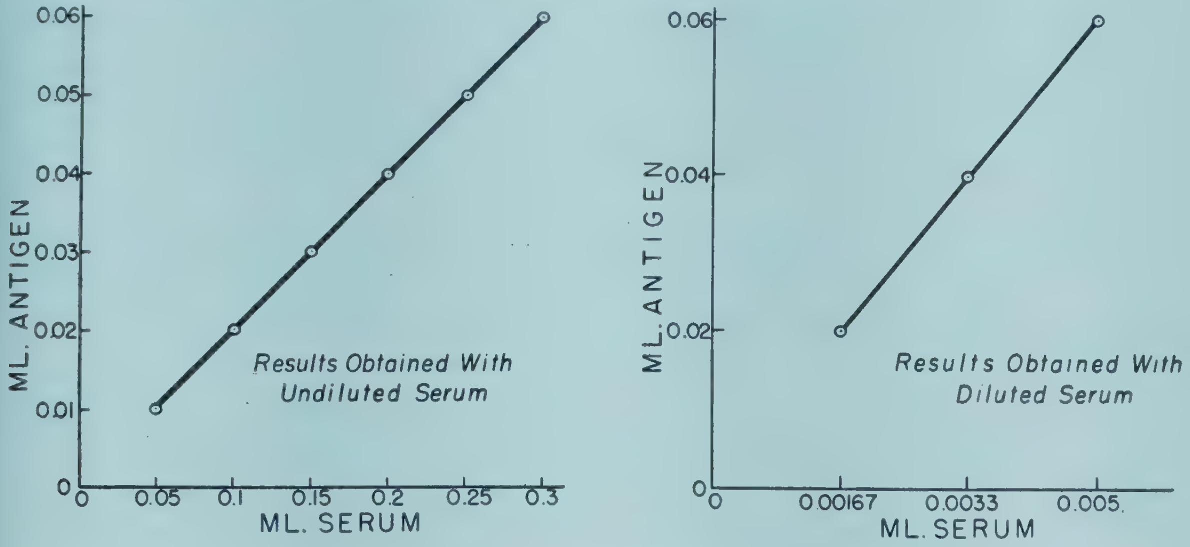


Fig. 52A. Proportional amounts of serum and antigen to give a 2+ reaction

Test with Undiluted Sera

Pipette 0.1 ml. of serum into each of three tubes (11- by 75-mm.) and add 0.02, 0.04, and 0.06 ml. of antigen suspension respectively. Shake the antigen suspension well before each pipetteful and wipe off the tip of the pipette on a piece of blotting paper. When the antigen has been added to the tubes of serum in one rack, shake vigorously by hand for five seconds making certain the antigen has mixed with the serum. Shake for three minutes in a machine of about 300 oscillations per minute and add 2.0 ml. of isotonic buffered salt solution (*F69A*). Mix thoroughly, let stand five minutes, and read. See table 59.

TABLE 59

The quantitative precipitation test with cardiolipin-lecithin-cholesterol antigen

TUBE NO.	1	2	3
	ml.	*ml.*	*ml.*
Serum	0.1	0.1	0.1
Antigen suspension	0.02	0.04	0.06
	Shake 3 minutes		
Salt solution*	2.0	2.0	2.0
	Let stand 5 minutes and read		

* Isotonic phthalate buffered salt solution (*F69A*).

To read the tests hold the tubes in the beam of light from a 60-watt daylight bulb against a black background. Record the results as −, ±, +, 2+, 3+, and 4+ according to the amount of precipitate and degree of clearing. It is necessary to have a slightly different standard for the three tubes because of the unequal concentration of particles. Use the minimum 2+ standards to determine the limit of significant degrees of precipitation. With some sera a prozone reaction may be evidenced by a different type of particulation characterized by the presence of some aggregates larger than others.

Centrifuge for five minutes at 2000 r.p.m. any tests in which an inhibited reaction (53) is suspected; that is, a low degree of reactivity in all tubes. Read by daylight. A coherent disc which does not break up on gentle shaking is read as 4+; the extent to which the disc breaks is recorded as 3+, 2+, +, and ±; and if the sediment redisperses on shaking, the reaction is read as −. Tests which require centrifugation are reported as atypical.

Test with Diluted Sera

Dilute sera that have titers greater than 5 in physiologic salt solution, preparing each dilution from the original serum. Not less than 0.02 ml. of serum should be pipetted for this purpose. The necessary dilutions for a given specimen should be selected from a series such as 1:2.5, 1:5, 1:10, 1:15, 1:20, 1:30, 1:40, 1:50, 1:60, 1:70, 1:80, 1:90, 1:100, 1:125, 1:150, etc. Perform a 1-tube test with each dilution of the serum using 0.1 ml. of the diluted serum and 0.02 ml. of antigen suspension. Use a fresh pipette for each dilution of serum. Do not pipette more than 6 doses of antigen suspension from one filling of the pipette. Shake three minutes and finally redisperse the test mixture with 2.0 ml. of isotonic phthalate buffered salt solution (*F69A*). The end point of the titration is the highest dilution to give a 2+ degree of precipitation.

Expression of Results of Tests as Titers

The use of different proportions of serum and antigen and of the minimum 2+ standard for reading makes it possible to express the results of tests accurately as titers (56). Table 60. The unit of evaluation is a 2+ reaction with 0.1 ml. of serum and 0.02 ml. of antigen. Thus, when a test reads 2+, +, ±, the titer of the serum is 1. Then a titer of 2 may be assigned to a serum when a 2+ reaction is obtained with twice this amount of antigen, 0.04 ml., and a titer of 3 when it is obtained with three times the unit dose of antigen, 0.06 ml. Data obtained from numerous tests of diluted sera (p. 460) provide a means for assigning titers above 3 (56). From the highest dilution giving a 2+ reaction median titers were calculated by the formula of Rider (57) and adjusted by dividing by $\sqrt{2}$. When the test read 4+, 3+, 2+ the adjusted median titer was calculated to be 3 which corroborates that obtained by use of the undiluted unit value. When the tests read 4+, 3+, 3+, and 4+, 4+, 3+ the adjusted median titers were 4 and 5 respectively. When tests read 4+, 4+, 4+ the titer may be considered to be greater than 5 and the serum must be tested in dilution if further quantitative information is desired. The highest dilution factor to give 2+ is considered the titer. While the titers as developed represent only an approximation of the reactivity of the serum they serve to meet the demand for a simple quantitative procedure.

TABLE 60

Expression of results of quantitative precipitation test as titers

REACTIONS WITH 0.1 ML. OF UNDILUTED SERUM			HIGHEST DILUTION FACTOR TO GIVE 2+	TITERS
ml. antigen				
0.02	0.04	0.06		
			(D)	
2+	+	±		1
3+	**2+**	+		2
4+	3+	**2+**		3
4+	3+	3+		4
4+	4+	3+		5
4+	4+	4+*		>5
			10	10
			15	15
			20	20
			etc.	etc.

* Prozone reactions such as 2+, 3+, 4+ are included in this group.

If readings of tests are intermediate, a range may be used to indicate the estimated titer. For example, 3+, +, − would suggest a titer of 1 to 2, or if a 3+ reaction is obtained when D = 15 and a + when D = 20, the titer would be expressed as 15 to 20.

Interpretation of the Test

Experience with the quantitative test is too limited to justify at this time a definite statement concerning an interpretation of the results, which should always be considered in the light of the clinical history and data. Titers of 5 or greater rarely have occurred in the absence of syphilis. Titers of less than 5 are believed to have diminishing diagnostic significance unless confirmed by history and corroboratory findings in other serologic tests. When a reaction is obtained only in the precipitation test a specimen should be sent for confirmatory examination.

THE PRELIMINARY PRECIPITATION TEST

The preliminary precipitation test is a 1-tube test using only the first mixture of 0.1 ml. of serum and 0.02 ml. of antigen suspension in the quantitative test described on p. 458. In setting up the tests, do not pipette more than 6 doses of antigen suspension from one filling of the pipette. Select for quantitative complement-fixation tests those sera which give a +, 2+, 3+, or 4+ precipitation reaction.

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CHAPTER 31

EXAMINATION FOR INCITANTS OF ACTINOMYCOSIS AND MYCOTIC DISEASE

Within recent years, the importance of actinomycotic and fungus infections has become more fully appreciated. While mycotic diseases, in particular, are especially prevalent in the tropics, some of the serious types of systemic infections are endemic in districts near the west coast of the United States. Ringworm infections of the scalp and hair among children in some of the larger cities have presented a particularly annoying problem and fungus infection of the feet is common in a large percentage of the population. Hence, while actinomycotic infections are still comparatively rare and most of the mycotic diseases do not produce serious systemic infections, laboratory aids in their diagnosis are of material value.

ACTINOMYCES

PUS

Microscopic Examination

If sulfur granules are present, collect one with a stiff dissecting needle, place on a glass slide, and apply a cover glass. Examine under low and high-dry objectives for the characteristic microscopic appearance of sulfur granules found in the lesions incited by actinomyces (1–4). Macerate another granule on a slide in a loopful of distilled water and stain by Gram's method (p. 35). Stain similar films by the Ziehl-Neelsen method (p. 43) and with aqueous alcoholic methylene blue (p. 38). Examine under a high-dry objective and an oil-immersion lens. While in cultures actinomyces are Gram-positive, in the sulfur granules parts of the structure may fail to retain the violet stain; hence the entire granule may not be Gram-positive. The majority of actinomyces are not acid-fast although some that grow under aerobic conditions are at least partly so.

When sulfur granules are not seen and the material is thick and tenacious, mount a loopful directly or in a sodium hydroxide-glycerol solution (p. 40). Examine under low and high-dry objectives for long, fine, branching filaments. Stain film preparations by the Gram and the Ziehl-Neelsen methods as well as with aqueous alcoholic methylene blue. Examine under an oil-immersion lens for long branching filaments, which are Gram-positive and, in most of the species of actinomyces, nonacid-fast.

Cultural Examination

Inoculate a loopful of the pus in duplicate in tubes of chopped meat medium (*F6*) and streak in duplicate in Petri plates blood agar (*F43A*) and beef-infusion agar (*F35*) with 1-per-cent glucose. Incubate one set under anaerobic and the other under aerobic conditions. Observe for at least two weeks. Do not inspect the anaerobic cultures oftener than at intervals of four to six days.

If growth occurs only in the anaerobic cultures, make moist preparations from the culture in liquid medium and from single colonies on solid medium and examine as directed under "Microscopic Examination." Freshly isolated cultures of *Actinomyces bovis* consist of long, thin, branching filaments; after artificial cultivation, the microorganisms may appear as pleomorphic bacilli resembling some of the corynebacteria. They are Gram-positive and nonacid-fast, and may be microaerophilic rather than strictly anaerobic.

If growth occurs only under aerobic conditions, stain films by the Ziehl-Neelsen method to differentiate the acid-fast and nonacid-fast species of the aerobic type (3). If the microorganism is nonacid-fast, consider *Actinomyces madurae* (*Nocardia madurae*) (5). Determine its identity by a study of the morphologic and cultural characters (2, 3). If acid-fast, inject guinea pigs to differentiate the species from the tubercle bacillus. For further identification, study the cultural and morphologic characters. Among the species of the aerobic acid-fast group, *Actinomyces asteroides* (*Nocardia asteroides*) has been reported to be pathogenic for man and *Actinomyces farcinicus* (*Nocardia farcinica*) for cattle and sheep—other species are also pathogenic for animals.

OTHER SPECIMENS

Examine such specimens as sputum, cerebrospinal fluid, urine, biopsy and postmortem material in the same manner as pus. For collection of the specimen, follow the directions on p. 469.

Actinomyces minutissimus (*Nocardia minutissima*, *Microsporum minutissimum*), the incitant of erythrasma (5–7), has rarely been cultivated on artificial media. As an aid in diagnosis, make sodium hydroxide-glycerol preparations (p. 466) of scrapings from the lesion and examine under high-dry and oil-immersion objectives for small actinomyces-like microorganisms. Also make stained preparations of scrapings with lactophenol-cotton blue (p. 40) or with aqueous alcoholic methylene blue (p. 38).

RINGWORM FUNGI (DERMATOPHYTES) AND FUNGI INCITING SYSTEMIC DISEASE

Specimens of skin, nails, hair, crusts, sputum, pus or other exudates, cerebrospinal fluid, feces, and tissue obtained at biopsy and postmortem examination may be studied for incitants of mycotic disease.

Special equipment needed for mycologic examinations consists of several long stiff dissecting needles, both straight and curved, and a long nichrome 24-gauge wire loop, in holders. Petroff flasks (see Fig. 53) or French square bottles should be used in preference to Petri plates to avoid the dissemination in the air of spores from some of the dangerous species.

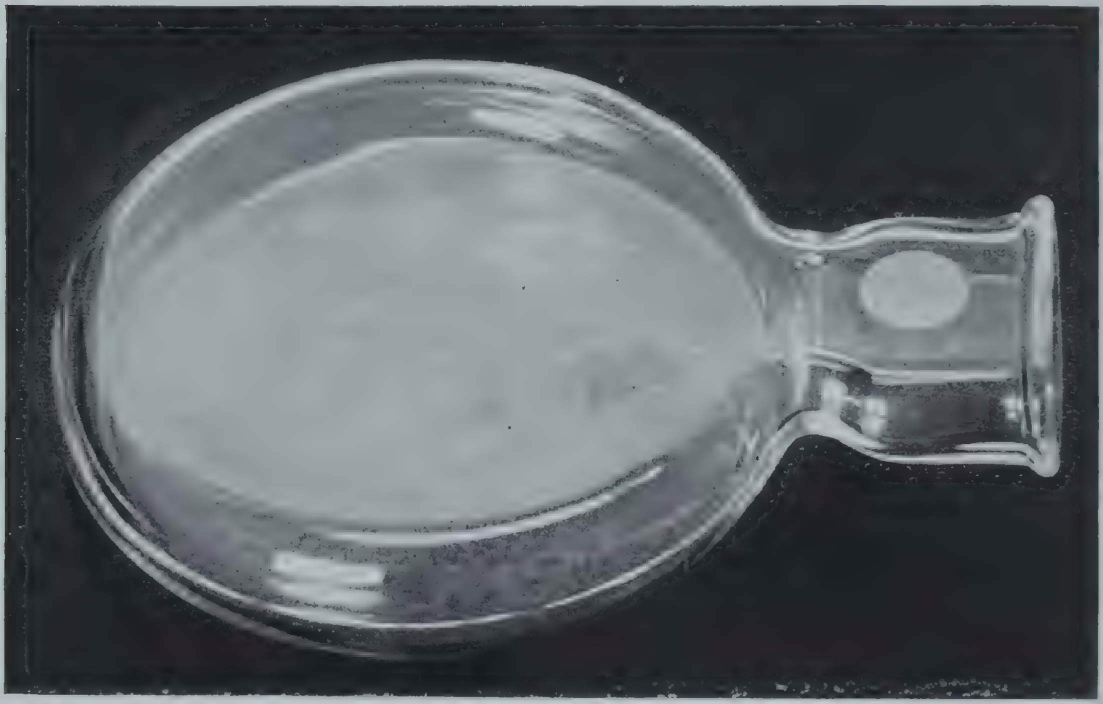

FIG. 53. PETROFF FLASK, 22 BY 95 MM.

Whenever the microorganism may be the incitant of a deep-seated infection, special precautions for handling cultures and specimens are required in mycologic studies in addition to those described for pathogenic bacteria (see p. 4). A draft-free room should be available, particularly for the work on cultures for identification, since the fruiting structures of fungi may easily become suspended in the air. It should be fully equipped with materials essential to the performance of mycologic studies. A large glass jar containing 10-per-cent cresol compound should always be available. Steel needles used in transferring infective materials should be sterilized before and after each use by heating to redness in a gas flame in the same manner as platinum needles and wire loops for bacteriologic work. Wear a gauze mask when working with a microorganism such as *Coccidioides immitis*.

SKIN, NAILS, AND HAIR

Collection of Specimen

Not only is the proper collection of a suitable specimen of the utmost importance, but especial care should also be given to the selection and preparation of the lesion. The material should be examined promptly after collection. Failure to find fungi does not necessarily exclude the presence of mycotic infection, since the material may not have been taken from the infected area or the fungus elements may have been destroyed in transit (6).

In addition to the usual equipment for bacteriologic work, small forceps (cosmetic), scalpels (Bard-Parker), small, sharp, curved scissors (manicure), and nail clippers should be available. The instruments should be soaked for at least thirty minutes in 10-per-cent cresol compound and placed in 70-per-cent ethyl alcohol saturated with sodium bicarbonate until ready for use and then wiped dry with a sterile towel.

The lesion should be washed thoroughly with 70-per-cent ethyl alcohol and wiped dry with a sterile gauze sponge to avoid as much saprophytic contamination as possible. Grease or excessive natural oil should be removed by rubbing the area with a gauze sponge soaked with benzene.

Smooth Skin.—Scrape the surface of the lesion with a scalpel. If it is widespread and has a well-marginated border, scrape material from the periphery. From ulcerative lesions involving the deeper layers of the skin or the subcutaneous tissue, collect the specimen from the underlying border with a scalpel, or if exudate is present, on a swab.

Feet and Hands.—With curved scissors remove the roof of fresh vesicles that are found in the interdigital spaces or sides of the fingers or toes, or on the plantar and palmar surfaces; obtain scrapings with a scalpel from around the edges of any fissure in the interdigital webs, preferably between the fourth and the fifth interspace on the foot. Scrape material from small slightly erythematous scaly patches on the toes, soles, heel, sides, and dorsa. If the interdigital webs of the hands or feet are involved, scrape the lesion with a scalpel or use sharp curved scissors. Collect scrapings with the scalpel from the deep layers of furrowed, discolored, distorted, crumbling, yellowish nails.

Scalp.—Examine the scalp of the patient under filtered ultraviolet light (Wood's lamp) for suitable areas from which to obtain a specimen. The microspora, chief incitants of tinea of the scalp, show a brilliant green fluorescence (a single infected hair may be detected by means of the fluorescence). Epilate with forceps several fluorescent hairs or, if the infection is slight, that is, only an occasional patch of alopecia with few stumps of hairs and scales is seen, scrape the areas with a scalpel.

The filtered ultraviolet light may be of little aid in selection of the more severe forms of scalp lesions incited by the trichophyta, since some of the species may show only a dullish white fluorescence, not always apparent. If fluorescent hairs are not found, examine the scalp also under a bright incandescent light, for alopecia and crusts. Remove crusts with forceps or scrape areas with a scalpel to obtain stubs of dry, gray hairs and crusts, while the patient is still under the light.

Disposition of Specimen.—Scrapings of skin and nails, hair, and crusts may be placed between two thoroughly cleaned, freshly flamed, glass slides or in a Petri plate. For mailing, the material may be enclosed in a glass tube or placed between flamed slides fastened together with adhesive tape, and submitted in a miscellaneous tube or slide outfit (see "Appendix C," p. 917, 921).

Microscopic Examination

Place a fragment of the scrapings from the skin or nails, or a few hairs or crusts on a glass slide with a straight dissecting needle. Cover with a drop or two of sodium hydroxide-glycerol solution (p. 40) and apply a cover glass. Warm in a flame and press the cover glass down until the specimen has been flattened. Allow a few minutes for clearing. Examine under low and high-dry objectives, adjusting the light to the proper intensity. If stained preparations are desired, use the method of Swartz-Conant with lactophenol-cotton blue (p. 40). Look for mycelium and spores in the case of skin and nails and for a sheath of spores in the form of a mosaic around the hair or for parallel rows of spores within the hair shaft. Repeat the examination if evidence of a mycotic infection is not found. Discard used slides and glassware into 10-per-cent cresol compound.

The preparation of semipermanent and permanent mounts is described on p. 41.

Cultural Examination

Isolation of the Fungus.—Prepare a sufficient quantity of Sabouraud's glucose and honey agar media (*F57.1B* and *F57.1C*) in tubes (150 by 19 mm.) to give a long slant and deep butt. With a dissecting needle, place a small amount of the scrapings from skin and nail or a few broken hairs and crusts, in a slight depression made with the point of the needle in the center of the honey and glucose agar slants. Incubate at room temperature and observe at frequent intervals for at least two weeks.

Make a microscopic study of any growth that occurs. Remove a small amount with a needle to a glass slide, cover with a drop of sodium hydroxide-glycerol solution (p. 40); if filamentous, tease apart and apply a cover glass. Examine under low and high-dry objectives for type of mycelium and spores. Structures such as septate mycelium, microconidia, macroconidia, and yeast-like microorganisms suggest pathogenic fungi and require further study.

The growth of pathogenic fungi is usually from white to cream in color, although a bright diffusible pigment may be obtained after long cultivation on some media. Saprophytic fungi (8, 9) are usually fast growing, highly pigmented, producing bluish-green or green, black, and yellow colonies. These characteristics help to distinguish them from pathogenic fungi. A few that are commonly found as contaminants (5–7) in cultures incubated at room temperature and that are easily recognizable by their cultural and morphologic characters belong to such genera as *Penicillium*, *Scopulariopsis*, *Asperigillus*, *Mucor*, *Cephalosporium*, *Alternaria*, and *Rhizopus*.

Identification of the Fungus.—Before attempting the cultural examination for identification of filamentous fungi, review the results of the microscopic examination of the original specimen and the clinical history. Among fungi that are classified as filamentous are those that, in the parasitic stage, appear as spherical or rounded bodies of varying dimensions and yet become filamentous in cultures. These fungi with a dual morphology are among those that incite deep-seated fatal infections in man (5–7).

Just before use, sterilize the necessary dissecting needles and loops by heating in a flame to a red glow and then place them horizontally on a rack, taking care to preserve their sterility. Allow sufficient time for cooling, since the instruments are thick metal. As one is used, sterilize and place on the far end of the rack for cooling.

Have Sabouraud's glucose and honey agars (*F57.1B* and *F57.1C*), corn meal agar (*F57.2*), wort agar (*F57.3*), and potato-glucose agar (*F56.8*) dispensed in Petroff flasks or French square bottles (100 or 200 ml.) placed horizontally. Inoculate the agar media and the rice medium (*F27.5*) with the primary culture. Remove a small amount of the growth with a dissecting needle to a small depression made in the center of the medium with the point of the needle. Incubate at room temperature for from ten days to three weeks before disturbing the growth.

Make sodium hydroxide-glycerol preparations (p. 466) of the growth on wort and corn meal agars and rice grains, and also stain a preparation with lactophenol-cotton blue (p. 40). Examine under low and high-dry objectives for macroconidia (fuseaux), that is, large asexual spores. Observe their shape and size, the number of septa, thickness of the cell wall, and whether they are smooth or rough, since the generic identity of the fungus is determined by the type found (10). Examine also for other important morphologic structures such as microconidia, their shape and size and whether sessile along the sides of the hyphae or in grape-like clusters; nodular bodies; racquet mycelium; spirals or coiled hyphae; pectinate (structures shaped like the teeth of a comb); hyphae; and chlamydospores (large spherical thick-walled cells).

Study the colonial characters of the fungus on the different media observing the color and type of mycelium, whether fluffy, sparse, granular, powdery, waxy, or velvety; whether the colony is fast- or slow-growing, smooth, heaped, folded, or grooved; and whether a diffusible pigment is produced, and, if so, the color.

The dermatophytes or ringworm fungi (10) are separated into three genera, *Microsporum*, *Trichophyton*, and *Epidermophyton*, according to the type of macroconidia produced. In *Microsporum*, these macroconidia are typically spindle-shaped with pointed tip, contain two to many cells, and are rough, and thick-walled; in *Trichophyton*, they are long, slender, rounded-end, contain two to several cells, and are smooth, thin-walled, and hyaline; and in *Epidermophyton*, large, clavate, contain few cells, and are smooth, thin-walled, and borne singly or in typical clusters. The species can be identified on the basis of certain morphologic structures and cultural characters (1, 5–7).

Tinea versicolor (pityriasis versicolor) (5, 6), a superficial type of skin lesion, is believed to be incited by *Malassezia furfur* (*Microsporum furfur*). Growth of the microorganism has not been obtained on artificial media. As an aid in diagnosis, make sodium hydroxide-glycerol preparations (p. 466) of scrapings from the lesion and examine under low and high-dry objectives for groups or clusters of round budding cells and fragments of mycelium.

To identify fungi with different morphology in the parasitic and cultural stages, inoculate the primary culture in duplicate on Sabouraud's glucose agar (*F57.1B*) slants and in the Petroff flasks or bottles as well as on blood agar (*F43A*) slants as described for dermatophytes. Incubate one set at room temperature and the other at 35°–37°C., and observe at intervals for at least four weeks. Important differential characteristics of fungi inciting deep-seated infections, such as blastomycosis, histoplasmosis, coccidioidomycosis, paracoccidioides (Almeida's disease), and chromoblastomycosis have been summarized by Benham (11, 12).

Blastomyces dermatitidis is the incitant of blastomycosis (American blastomycosis or Gilchrist's disease). This fungus appears in pathologic material as a large, spherical, thick-walled, budding, yeast-like microorganism. On blood or Sabouraud's glucose agar incubated at 37°C., it retains the yeast-like appearance; on glucose agar incubated at room temperature, the growth is filamentous. The microorganism has been found to be pathogenic for dogs and monkeys and to a less degree for guinea pigs.

Histoplasma capsulatum, the etiologic agent of histoplasmosis (13–18), is a small oval or budding cell in the parasitic stage (5), particularly in the monocytes. On blood agar incubated at 37°C., it is yeast-like; at 22°C. on Sabouraud's glucose agar, the growth is mycelial, the colony white and downy with characteristic spores. To establish a diagnosis of histoplasmosis, make films from the peripheral blood, sternal bone marrow, or material from a lymph node puncture, stain by Giemsa's method (p. 34), and examine for small oval, budding cells in the monocytes. Mice, rats, guinea pigs, dogs, and monkeys are reported to be susceptible to infection with this microorganism.

Coccidioides immitis, the incitant of coccidioidomycosis, ("California disease," "valley fever," and coccidioidal granuloma) (19, 20), in pathologic materials has large, thick-walled, spherical cells with endospores. On culture media incubated at room temperature, the mycelial growth is white and fluffy. Microscopically, the culture shows numerous arthrospores (rectangular, elipsoidal, rounded cells formed by the fragmentation of hyphae). Laboratory animals, particularly the guinea pig, have been found to be highly susceptible to infection with this microorganism. This characteristic is of value in differentiating *Coccidioides immitis* from *Blastomyces dermatitidis*. Complement-fixation and skin tests have been reported to be of value as aids in diagnosis (21).

Paracoccidioides brasiliensis, the etiologic agent of paracoccidioides (Almeida's disease) (22, 23), is found in tissue or pus as large, thick-walled, spherical cells with multiple budding. Cultures grown on Sabouraud's glucose and blood agar slants at 37°C. have a yeast-like morphology; at 20°C., round or oval chlamydospores with multiple budding and scanty mycelium. The microorganism has been found to be slightly pathogenic for laboratory animals, rats being the most susceptible.

Phialophora verrucosa, *Hormodendrum pedrosoi*, *Hormodendrum langeroni*, *Hormodendrum compactum* have been designated as the incitants of chromoblastomycosis (verrucous dermatitis) (11, 24–26). They appear as round, thick-walled, dark-brown bodies within giant cells of tissue. The differentiation of the species is based on the results of cultural studies.

On Sabouraud's glucose medium at room temperature, they are slow-growing, dark green to black, felt-like in appearance, and only slightly embedded in the medium and easily dislodged. Microscopically, three types of sporulation are recognized: *Phialophora* is characterized by cup-shaped conidiophores (specialized hyphae that bear conidia, asexual spores) with spores that bud from the base of the cup; *Hormodendrum*, by tree-like branching conidiophores with conidia in chains; and *Acrotheca*, by conidiophores in the form of a terminal cell or lateral branch that becomes swollen and from which conidia are borne on short protuberances. All three types are reported to have been found in a single spore strain of one fungus (11, 25). The question has therefore been raised whether these agents of chromoblastomycosis represent closely related or identical species. Laboratory animals have been found to show little susceptibility to these strains.

Sporotrichum (Rhinocladium) schenckii (beurmanni) is the etiologic agent of sporotrichosis. The fungus is seldom found in film preparations made directly from the pus of the lesion. A definite diagnosis can usually be established by cultural examination of the material from the lesion and by animal inoculation. On Sabouraud's glucose and blood agar slants incubated at room temperature and at 37°C., the colonies are cream-colored and smooth, later becoming corrugated and turning from dark-brown to black. Unlike most other fungi, this microorganism forms no aerial mycelium on the usual media. White male rats inoculated intraperitoneally with a heavy suspension of the suspected material develop a purulent orchitis and films made from the exudate will usually show the characteristic Gram-positive cigar-shaped fungi.

Monosporium apiospermum is the most common incitant of maduromycosis (madura foot, mycetoma). Pus, scrapings, or biopsy tissue from lesions should be examined grossly for granules and studied as directed under "Actinomyces." The granules are composed of a mass of segmented, branched hyphae and generally chlamydospores (5, 27). The growth of *Monosporium apiospermum* on Sabouraud's glucose agar at room temperature is gray and feathery with branching mycelium and a blackened undersurface. Microscopically, many ovoid pyriform microconidia are found singly or occasionally in groups along the hyphael threads and attached by sterigmata. Animal inoculation is of no aid in diagnosis. *Actinomyces (Nocardia) madurae* is sometimes found as the incitant of madura foot or mycetoma. The granules in lesions produced by this microorganism have the same characteristics as those in actinomycotic infections (5).

Among yeast-like fungi are two well-known pathogens, *Candida (Monilia) albicans* and *Cryptococcus neoformans (hominis) (Torula histolytica)* as well as a large number of yeasts and yeast-like fungi that occur as contaminants and from which the pathogenic forms need to be differentiated.

Inoculate corn meal agar (28) (*F57.2*) in Petroff flasks or bottles with the primary culture by cutting through the agar along the line of streak. A 24-gauge wire loop bent at right angles is useful for the purpose. Incubate at room temperature for one or more weeks. The presence of mycelium along the line of streak differentiates the *Candida* group from the *Saccharomyces* and *Cryptococcus*. If present, examine under a low-power objective. Also make a sodium hydroxide-glycerol preparation (p. 466) from the mycelial growth cut from the agar. Examine under low and high-dry objectives for chlamydospores (large spherical thick-walled cells) and for clusters of budding cells, which are characteristic of *Candida albicans*.

For further differentiation of this fungus (5, 28, 29), study the fermentative properties and test the pathogenicity for rabbits. Employ peptone medium (*F22*) with Andrade's indicator and 1-per-cent glucose, maltose, sucrose, and lactose, dispensed in Durham's tubes. Rabbits inoculated intravenously with 0.2 ml. of a 1:100 suspension of *Candida albicans* in salt solution usually die between the fourth and fifth day. Multiple abscesses are found in the cortex of the kidney.

If no mycelium is present on the corn meal agar plate, make a sodium hydroxide-glycerol preparation from the culture and examine under low and high-dry objectives for ascospores (spores borne in a sac-like cell). *Cryptococci* can thus be differentiated from the *Saccharomyces* since the latter produces ascospores and the former do not. If ascospores are not found, inoculate in duplicate Sabouraud's glucose agar (*F57.1 B*) and blood agar (*F43A*) slants with the culture. Incubate one set at room temperature and the other at 35°–37°C. and observe frequently for two weeks. Suspend some of the culture in India ink on a glass slide. Apply a cover glass and examine immediately for thick capsules; also stain a preparation by Gram's method (p. 35).

Cryptococcus neoformans (*hominis*) (*Torula histolytica*) forms Gram-positive spherical or oval budding cells with wide capsules. It grows readily at 22°–37°C. (30) and is pathogenic for laboratory animals. The saprophytic forms will grow poorly or not at all at 37°C. and are not pathogenic.

To determine pathogenicity, inject rats intraperitoneally with 1 ml. of a 1:100 suspension of the culture in salt solution. Kill and autopsy the animal between the second and third weeks after inoculation. Look for small white nodules on the spleen particularly, the liver, kidneys, and the lungs. Make cultures from the lesions on Sabouraud's glucose agar. (The microorganism should be recovered.) Make sodium hydroxide-glycerol and India ink preparations from a small amount of the macerated tissue showing the presence of nodules. Examine under low and high-dry objectives for round budding cells, adjusting intensity of the light so as to reveal the wide capsule surrounding the microorganism. Prepare also films from tissue and stain by Gram's method (p. 35). Examine under an oil-immersion lens for round budding cells with wide capsules.

SPUTUM

When a pulmonary infection is suspected, sputum should be obtained with a bronchoscope, whenever possible. Otherwise, several specimens collected from the deeper air passages and obtained on different days should be examined.

Microscopic Examination

Mount a large loopful of the material directly on a glass slide or, if the specimen is thick and tenacious, make a sodium hydroxide-glycerol preparation (p. 466) and warm gently in a flame. Examine under low and high-dry objectives for small round or oval budding cells, or large spherical cells with endospores. The light should be carefully adjusted to give the proper intensity. Look also for sulfur granules as described under "Actinomyces," p. 466. Examine films stained by Gram's method, with aqueous alcoholic methylene blue (p. 38), or crystal violet (p. 37).

Cultural Examination

Inoculate Sabouraud's glucose agar media (*F57.1 B*) in a Petroff flask by taking up the specimen on a long, slightly curved wire loop and touching it to the medium at several points so that growth will occur in several contact points; also place a loopful of the specimen in the center of the glucose agar and blood agar (*F43A*) slants. Prepare duplicate cultures and incubate one at room temperature and the other at 35°–37°C. for at least four weeks. Observe at frequent intervals. Study as outlined under "Skin, Nails, and Hair," p. 469.

PUS

Pus from palpable glands and from cutaneous or subcutaneous abscesses should be aspirated with a syringe and needle under aseptic precautions and placed in a tube. Material from the suppurating lesion should be collected on two or more sterile swabs. Examine as outlined under "Sputum."

EXUDATE

Exudate from lesions on the mucous membranes should be collected on swabs by rubbing the swab over the lesion. Emulsify the material on a swab in honey broth (*F22.5*) and examine the suspension as directed under "Sputum." Then incubate the inoculated medium for from three to five days and examine.

CEREBROSPINAL FLUID

Examine as described in "Examination of Cerebrospinal Fluid," p. 517. In addition, place a drop of the sediment on a slide, apply a cover glass, and examine under low and high-dry objectives. If round, budding cells are present, suspend a drop of sediment in India ink and examine immediately for thick capsules. Stain a film preparation by Gram's method (p. 35) and one with aqueous alcoholic methylene blue (p. 38). Proceed with the cultural examination as directed under "Sputum," p. 475.

FECES AND URINE

Submit specimens of feces and urine promptly after collection and without preservative. Urine should be collected with aseptic precautions. Centrifuge the urine. Examine the sediment and feces as directed under "Sputum."

TISSUE

Observe precautions for the handling of pathogenic fungi (p. 4). Tissue from biopsy or post-mortem examination should be collected aseptically and submitted promptly without preservative for cultural and histologic study. If the examination cannot be made immediately after collection and sufficient tissue is available, a small portion should be placed in Zenker's solution (p. 550) or formalin (p. 547).

For cultural examination, grind a portion in a mortar with sand and examine as directed for specimens from skin lesions. For histologic study, embed a portion in paraffin, cut thin sections, and stain by Gram's method (p. 35) and by the hematoxylin-phloxine or hematoxylin-eosin method (p. 550).

Place all contaminated utensils and slides in a 10-per-cent cresol compound and autoclave at 121°C. for thirty minutes before processing for future use.

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CHAPTER 32

EXAMINATION FOR PROTOZOA (1–6)

Diseases incited by protozoa have been in the past of minor importance in temperate sections of the United States, provided the sanitary environment was satisfactory. Now, however, laboratory aids in the diagnosis of these infections must be readily available, in view of the return of those who have been in military service in regions where protozoan diseases are prevalent. Specimens should preferably be examined promptly after collection. Pertinent data in regard to the history of the patient are of particular importance since information concerning the geographic distribution of the various pathogenic species when correlated with clinical manifestations may furnish valuable clues in regard to the laboratory examinations that will be of greatest value.

For practical purposes, protozoa of medical importance may be divided into two groups: those that parasitize the gastrointestinal tract and also the vagina, and those that are found in the blood and tissue.

INTESTINAL PROTOZOA

Several species of amebae, flagellates, and ciliates inhabit the gastrointestinal tract. However, only *Endamoeba histolytica* and *Balantidium coli* are generally recognized as pathogenic for man. *Balantidium coli* is only rarely found. Since various types of amebae resemble each other closely, their differentiation is of the utmost importance.

AMEBAE (7, 8)

The term amebiasis includes all clinical types of infection with *End. histolytica*, while amebic dysentery applies strictly to infections in which there are lesions of the intestines and diarrhea and bloody stool. A diagnosis of amebiasis can only be established by finding *End. histolytica* in feces, exudate, or tissue. In the fluid stool from patients with acute amebic dysentery, usually only the vegetative form or the trophozoite is found. In the semiformed stool, either trophozoites or cysts may be present while in the formed stool only cysts are found. However, when mucus from an amebic ulcer is adherent on the surface of a formed stool, trophozoites may be demonstrated. Failure to find *End. histolytica* in a single stool does not exclude amebiasis, and it may be necessary to examine at least six stools collected on alternate days.

Collection of Specimen

All specimens should be examined promptly after collection. A delay of even an hour usually renders the examination for trophozoites of amebae unsuccessful. Cysts can often be found in specimens forty-eight hours old and may be preserved in 4- or 5-per-cent formalin. When prompt examination for trophozoites is not possible, films may be prepared as for Heidenhain's method (p. 482) and mailed to the laboratory in 70-per-cent alcohol. This procedure is important since cysts are relatively rare in fluid stools containing trophozoites.

A satisfactory specimen is of the greatest importance. Feces should be collected in a clean, dry, metal or glass container sterilized by heat rather than antiseptic, and preferably warmed to body temperature. The specimen should be free from water or urine. The patient should preferably come to the laboratory and pass a stool so that it can be examined while still fresh. If he cannot come to the laboratory, he should be hospitalized and the specimen collected as described. The whole stool should be examined macroscopically and portions showing bloody mucus or mucus selected for microscopic study. Fluid stools sent through the mail are not satisfactory for examination since they contain only motile forms that degenerate within an hour after collection of the specimen. Films may be prepared from blood-stained particles, however, and mailed to the laboratory in 70-per-cent alcohol. If the stools are formed, a small portion (20–30 grams) can be placed in a 2-oz. glass jar and sent through the mail to be examined for cysts.

Purulent exudate from ulcers and abscesses and specimens collected through a proctoscope from ulcerated areas in the intestinal mucosa must be examined promptly after collection since only trophozoites are present. Material obtained by proctoscope, however, is desirable only after failure to demonstrate *End. histolytica* in stools. If the examination cannot be undertaken immediately, films may be prepared and sent to the laboratory in 70-per-cent alcohol.

Microscopic Examination

The cellular elements in amebic and bacillary dysentery vary markedly (9, 10). In amebic dysentery, the exudate consists chiefly of mucus, a few degenerated pus cells, and red blood cells, frequently in clumps. Charcot-Leyden crystals may also be present. In bacillary dysentery, there is a profuse exudate consisting chiefly of polymorphonuclear leucocytes and large macrophagic cells.

Trophozoites, varying in size from 15–16μ, with characteristic linear motion and containing red blood cells may be regarded as *End. histolytica.*

The cysts of this protozoan are 6–20μ, thin-walled, and may have from 1 to 4 nuclei with a polar arrangement; those of *Endamoeba coli* are 10–33μ and have from 1 to 8 nuclei centrally and irregularly arranged. The nucleus of the *End. histolytica* cyst has a finely granular periphery and a caryosome that appears as a very minute refractile dot at the center of the nucleus; that of *End. coli* is heavily and irregularly outlined with an eccentric caryosome.

The trophozoites of *Endolimax nana* are 6–15μ and may resemble in size small races of *End. histolytica.* Hence, specimens containing any small trophozoites must be stained to verify their nuclear character. The cysts of this protozoan are 5–14μ, oval, and contain 1 to 4 nuclei with large caryosomes; chromatoidal bodies are usually absent. The trophozoites of *Iodamoeba butschlii* are rarely seen; the cysts are 5–20μ, odd-shaped, uninucleate, and without chromatoidal bodies but contain a characteristic glycogen mass. The trophozoites of *Diendamoeba fragilis*, which are often very difficult to stain, are 5–12μ, with a circular outline; cysts are not known to form. The genera *Iodamoeba*, *Diendamoeba*, and *Endolimax* are believed to be represented by only one species each.

Fresh Film Preparations.—Place a portion of feces about 2 mm. in diameter in the center of a clean slide and emulsify with a large drop of salt solution. Apply a cover glass and examine under a microscope, using a low magnification. By this method, the motile as well as the encysted forms of amebae can be studied. When searching for trophozoites, a warm stage is desirable. Trophozoites can be recognized by their motility; cysts appear as highly refractile round or oval bodies. Details in regard to the morphology of the various amebae can best be obtained from standard texts (2a, 4b, 11, 12).

Aqueous Film Preparations (13).—Place a portion of feces 2 mm. in diameter in the center of a clean slide and emulsify with a drop of fresh tap water. The tap water destroys *Blastocystis hominis* and thus facilitates the search for amebic cysts. This method is also useful in differentiating *Diendamoeba fragilis.*

Stained Preparations.—*Methylene Blue-Cadmiun-Sudan* (*Quensel*) (14). Place a portion of feces 2 mm. in diameter on a slide, mix it with a large drop of Quensel's stain (p. 39), and apply a cover glass. After from fifteen to twenty minutes, the trophozoites are stained pale blue with the nuclei a darker shake. The nuclei of *Diendamoeba fragilis* do not stain, but the other amebic species show the same nuclear structures as when stained by the Heidenhain iron-hematoxylin method. Amebae can also be easily distinguished from *Blastocystis* since in the latter the peripheral layer of cytoplasm with enclosed nuclei is clearly recognizable even under a low magnification.

Iodine.—Place a portion of feces 2 mm. in diameter on a slide, mix with a large drop of D'Antoni's or Lugol's iodine solution (p. 32 or p. 37) and apply a cover glass. Set aside for at least five minutes to allow the iodine solution to penetrate the cysts. The iodine stains the cysts light brown, clearly demonstrating the nuclei and the characteristic caryosome. This procedure is satisfactory for cysts only.

Concentration Method for Cysts.—When amebae or their cysts are not found and the specimen is from a formed or semiformed stool, examine by the zinc sulfate flotation method of Faust (15, 16). Dilute 1 part of feces in 10 parts of lukewarm salt solution or tap water. Strain through one or two layers of wet gauze. Collect the filtrate in a centrifuge tube of approximately 15-ml. capacity. Centrifuge the filtrate for from forty-five to sixty seconds at 2500 r.p.m. Decant the supernatant and add water or salt solution until the tube is nearly full. Thoroughly distribute the sediment throughout the tube. Centrifuge as before and repeat until the supernatant is relatively clear. Decant the supernatant and add sufficient zinc sulfate solution (p. 44) to fill the tube to within one-half inch of the rim. Centrifuge for ninety seconds at 2500 r.p.m. By means of a wire loop bent at a right angle, carefully transfer several large loopfuls of the material from the surface of the zinc sulfate solution to a glass slide, add a drop of iodine solution (p. 32 or p. 37), apply a cover glass, and examine for cysts.

Heidenhain's Iron-Hematoxylin Method.—For confirmatory examination, fix and stain preparations by Heidenhain's iron-hematoxylin method. Do not use metal instruments. Be sure to keep preparations moist throughout the entire staining procedure. Make thin films of emulsified feces on clean slides with a small brush or applicator. While still moist, immerse films in Schaudinn's solution (see p. 549) at room temperature or at 37°C., twenty to thirty minutes. Transfer to 70-per-cent alcohol for a few minutes.

Place slides in 70-per-cent alcohol containing sufficient iodine to give it a port-wine color, fifteen to twenty minutes. Transfer to 70-per-cent alcohol, five minutes or more. Films may remain in 70-per-cent alcohol at this stage for a considerable period and may be shipped in it to a laboratory to be stained.

Wash in running tap water for a few minutes and rinse in distilled water. Place in a mordant of 4-per-cent aqueous iron-alum solution (p. 36), one to twelve hours. Wash in running tap water for a few minutes to remove excess of iron alum and stain in 0.5-per-cent Heidenhain's hematoxylin (p. 36), approximately twelve to eighteen hours. Wash in running tap water, five minutes. Differentiate in a 2-per-cent aqueous iron-alum solution. Control the degree of differentiation with the microscope. This is the most important step and requires great care. At intervals, remove the films from the iron-alum solution, wash in running tap water for a minute, and examine moist with the microscope. When the films are sufficiently decolorized so that the nuclei are black and the cytoplasm gray, stop the further differentiation by transferring the slides to tap water. Wash in running tap water, one to two hours. Dehydrate by passing the slides through ascending grades of alcohol from 70 per cent through 95 per cent and two changes of absolute alcohol, five minutes in each. Place in two changes of xylol, five minutes in each. Mount in neutral xylol-dammar (p. 548).

When necessary to save time, the preparations may be fixed at 60°C., Heidenhain's hematoxylin heated to 40°C., and the various staining procedures shortened to approximately two to five minutes (17). Make thin films, fix in Schaudinn's solution at 60°C. for ten minutes. Transfer to 95-per-cent alcohol to which enough iodine solution has been added to give a port-wine color. Leave for five minutes. Transfer to 70-per-cent alcohol, five minutes. Transfer to running tap water, two minutes. Place in 4-per-cent aqueous iron-alum solution for fifteen minutes; transfer to running tap water, two minutes. Stain with 0.5-per-cent aqueous hematoxylin for ten minutes. Differentiate in 0.25-per-cent aqueous iron-alum solution for twelve minutes. Wash in running tap water, thirty minutes. Dehydrate by passing through ascending percentages of alcohol, 60-per-cent, 70-per-cent, 90-per-cent, 95-per-cent and absolute alcohol, two minutes each. Clear in xylol and mount in neutral xylol-dammar.

Tompkins and Miller (47) recommend the following rapid method. Fix thin films in Schaudinn's solution at room temperature for five minutes. Transfer through 70-per-cent alcohol containing sufficient iodine to give a port-wine color, then through 70-per-cent alcohol to 4-per-cent aqueous iron-alum solution and leave for three to five minutes. Wash in running tap water, one minute. Stain with 0.5-per-cent hematoxylin for one minute only. Dip in tap water to remove the stain and differentiate in 2-per-cent phosphotungstic acid aqueous solution for two to five minutes. Wash in tap water. Dehydrate and mount.

Cultural Examination

For cultural studies of feces for *End. histolytica*, only freshly passed fluid or semifluid specimens are suitable. Two types of media have been found satisfactory for this purpose—egg albumen medium (*F16*) and asparagine serum agar medium (*F54*). In both, the medium consists of a solid substratum overlayered with an albuminous liquid. If two parts of rice starch and one of animal charcoal are added to the liquid portion of the medium before seeding it, the cultures may remain viable for four days.

Add about 0.5 ml. of fluid feces to the liquid portion of the medium, mix well, and incubate for twenty-four hours. The amebae are found in greatest concentration at the junction of the solid and liquid layers. Use a capillary pipette with an aperture at the tip about 2 mm. in diameter to obtain the material. Examine a large drop. If amebae are not found, study three or four preparations. Also, examine all preparations again after forty-eight hours. Transfer subcultures at 48-hour intervals. The amebae can be stained with Heidenhain's iron hematoxylin for confirmation.

FLAGELLATES

A large number of flagellates inhabit the digestive tract, the most important of which are: *Chilomastix mesnili*, *Trichomonas hominis*, and *Giardia lamblia*. *Giardia* have been reported to be pathogenic, particularly in children (18–20); many patients have symptoms of gastroenteritis that is relieved by specific therapy.

The trophozoites of these flagellates are sufficiently characteristic in structure to be easily identified. *Giardia lamblia* travels with a slow tumbling motion; *Chilomastix mesnili* has a torpedo-like movement; and *Trichomonas hominis* can be recognized by its undulating membrane and lashing flagella, both of which may be lost when old. The trophozoites of *Giardia lamblia* are 12–15μ long, pear-shaped anterior-posteriorly, and spoon-shaped laterally. They have "spectacled" nuclei, parallel axostyles, and tails. The cysts are football shaped with a narrow clear space between the wall and encysted microorganism. *Chilomastix mesnili* trophozoites are cone-shaped, usually 12–15μ long, occasionally 5–8μ. The cell contains a spiral groove, which is an identifying character. The cysts are 8μ long, lemon-shaped with a nipple-like thickening of the wall at the narrower end. The cytoplasm is dense and there is no trace of flagella or nucleus.

Trichomonas hominis resembles *Trichomonas vaginalis* in morphology and is not known to form cysts.

For detailed descriptions of these protozoa, consult standard texts (2b, 4b, 11, 12). It is important not to confuse the cysts of flagellates with those of *End. histolytica*. For the microscopic examination of fecal specimens, follow the same procedures outlined for *End. histolytica*.

Trichomonas vaginalis (21) is a flagellated protozoon not infrequently found in leucorrheal discharge. Its exact etiological significance remains unsettled. Many gynecologists consider it mildly pathogenic and describe a clinical syndrome of trichomonas vaginitis characterized by profuse leucorrhea and mild irritation of the affected part. A diagnosis is established by demonstrating the protozoa in the discharge.

Moist preparations of freshly collected material are preferable to dried films. Place a drop of the material, preferably diluted with an equal amount of salt solution, on a clean slide or cover glass and examine under a high-dry objective with the iris diaphragm of the microscope adjusted to exclude most of the light. In fresh material, *T. vaginalis* is exceedingly active and easily recognized by its motion and the flagella. This method can be undertaken only when the patient is available and the material can be examined promptly. When this is not feasible, make at least four slide preparations in the same manner as blood films for a differential leucocyte count. They must be very thin, made quickly, and dried rapidly. Stain some for *T. vaginalis* by Wright's method (p. 42); the others for *Neisseria gonorrhoeae* by the Gram method (p. 35).

If the films are properly made, the protozoon is easily recognized. It is a pear-shaped microorganism about two to four times as large as a white blood cell. The cytoplasm appears pale blue and contains a reddish spindle-shaped nucleus with a bright red blepharoplastic granule at the anterior end. The flagella are seldom stained by the Wright method.

CILIATES

Balantidium coli (22, 23) is frequently present in the feces of hogs and is the only ciliate that has been found to be pathogenic for man. The clinical symptoms of infection with this protozoan are very similar to those of amebic dysentery and a diagnosis can only be established by demonstrating *Balantidium coli* in stool or in tissue. Follow the microscopic procedures outlined for *End. histolytica.* The microorganism is easily recognized by its large size, 50–70μ long, and characteristic appearance. It is oval with wavy rows of cilia and darts about in a slow-rotating motion, contains a cyclostome or mouth, a reniform macronucleus and a smaller micronucleus as well as two contractile vacuoles. The cysts, which are rarely seen, are 50–60μ long, round, thick-walled, have nuclei, and contractile vacuoles.

PROTOZOA IN BLOOD AND TISSUE

Protozoa found in the blood and tissues include *Plasmodium* of malaria, *Trypanosoma*, and *Leishmania.*

PLASMODIUM OF MALARIA

Four species of malaria parasites are recognized: *Plasmodium vivax*, the incitant of tertian malaria; *Plasmodium malariae*, quartan malaria; *Plasmodium falciparum*, malignant tertian (estivo-autumnal) malaria; and *Plasmodium ovale*, an infection similar clinically to tertian malaria (24, 25). A diagnosis of malaria must depend upon demonstration of the parasite in the blood or tissues. Identification of the species is important since the clinical symptoms, especially in falciparum malaria, may present an acute medical emergency.

Blood

Both thick and thin blood films should be prepared from twelve to twenty-four hours after a chill. Recent studies advise films every six hours until the diagnosis is established (27). By means of the thick film, the presence of malaria parasites is determined; by the thin film, the types are identified.

Bass and Johns (26) have recommended a concentration method for demonstration of the parasites in citrated blood.

Thick Blood Films.—Place 3 to 5 average-sized drops of blood about one-fourth inch from the end of the slide. These may be deposited on an area about three-quarters of an inch in diameter and promptly combined with a needle or the corner of another slide; or the surface of the slide may be touched to a large drop and the slide moved in narrow circles until a preparation of the size mentioned is obtained. The film must be fairly thick in order to have sufficient blood for adequate examination but must not be so thick that it will peel from the slide when it is being stained. When the thick blood film is still wet, ordinary printing can just be read through it. Lay the slide flat to dry so that the blood may be evenly distributed, and protect it from dust by supporting it in an inverted position or by placing it in a covered Petri plate. Allow eight to twelve hours for drying unless the process can be hastened by placing it for a few minutes in an incubator at 37°C. or by using an electric fan. If the fan is used, avoid stirring up dust or causing ripples in the drying film. Stain by Giemsa's method (p. 34). Consult studies by Wilcox (28) and Field (29, 30) for morphology of the parasites in thick films.

Field (31) has recommended an accelerated modification of his original staining procedure which is also reported to have given excellent results in laboratories of the military service (27, 34). Prepare thick films in the usual manner. Stain (p. 33) as soon as they cease to be moist. Dry in the air. Do not fix.

The procedure recommended by Michelson and Wilcox (32) will also save time.

Thin Blood Films.—*Plasmodium vivax* and *P. malariae* can best be differentiated in films made after the first twenty-four hours of the asexual cycle; *P. falciparum*, during the first twenty-four hours after a chill.

Make blood films as directed under "Examination of Blood," p. 505. Stain by Giemsa's method (p. 34).

In infections with *P. vivax*, all stages of the asexual cycle develop in the peripheral blood. Therefore trophozoites or ring forms, ameboid forms, as well as gametocytes are found. The red cells are enlarged as the parasites develop in them and usually show numerous fine eosinophilic granules known as Schüffner's dots. The trophozoites or ring forms have thick cytoplasm stained blue with a deep red chromatic granule. Generally only one parasite is found in a cell although occasionally two may be present. As the trophozoites develop, they appear as irregular ameboid forms and the red blood cells containing them are distinctly enlarged. The mature schizont usually consists of sixteen or more merozoites. The pigment is light brown and tends to clump towards the center. The gametocytes are generally spherical and almost completely fill the red cell.

In infections with *P. malariae*, all stages of schizogony are present in the peripheral blood. The red cells are not enlarged. This characteristic is an important means of differentiating the species from *P. vivax*. The trophozoite or ring form is found infrequently. The most characteristic stage of the parasite is the so-called band form which stretches across the entire diameter of the red cell. When merozoites are present, there may be 6 to 12, but most commonly only 8. They have a characteristic "daisy" appearance and do not completely fill the erythrocyte.

The red cells in infections with *P. falciparum* are usually not enlarged. The trophozoite or ring form and the gametocyte are the only types generally seen in the peripheral blood. The ring forms are more numerous, smaller, and more delicate than those of *P. vivax*. Frequently two or more parasites are present in a single cell. Maurer's dots may occasionally be found in the red cell. The gametocytes are crescent-shaped and, when found, leave no doubt as to the identity of the parasite. They can often be readily recognized in thick films. However, they may be distorted, folded, or on end, in which case they may be mistaken for band forms of *P. malariae*. The chromatin and coarse brown pigment are diffusely scattered through the central portion of the gametocyte.

Infections with *P. ovale* are relatively uncommon.

Table 61 summarizes the chief characteristics of three types of malaria parasite. Illustrations of their appearance are also helpful (2b, 3a, 4c, 6a).

Combined Thick and Thin Blood Films.—Make a thin film at one end of the slide in the usual manner and a thick film starting one-half inch from the thin film. It is well to separate the two by a waxed-pencil line. After the thin film is dried in the air, fix that portion for three or four minutes by standing the slide upright in a jar with sufficient absolute methyl alcohol to immerse the slide up to the waxed line. Still holding the slide verticle, remove from the alcohol and allow to dry in that position. Then dry the thick film in the usual manner and stain the entire slide with Giemsa's method in the same manner as for thick films. Recently, Field's method for staining thick films has been adapted for combining thick and thin films (33, 34).

Bone-Marrow Films.—Occasionally sternal puncture may be advisable.

Prepare films of the bone marrow on glass slides and stain by Giemsa's method in the usual manner for thin films.

Tissue

Fix tissue in Zenker's solution and stain by Giemsa's method (p. 552). Other methods are recommended by Tomlinson and Grocott (35) and Lendrum (36).

TABLE 61

Chief differential characteristics of malaria parasites

	Plasmodium vivax	*Plasmodium malariae*	*Plasmodium falciparum*
Erythrocytes containing parasites, enlarged	+	−	−
Schüffner's dots	+	−	−
Large irregular rings	+	−	−
All forms in peripheral blood	+	+	−
Band forms	−	+	−
Maurer's dots*	−	−	+
Double chromatin dots	−	−	+
Multiple infection	Rare	−	+
Crescents	−	−	+
Pigment abundant and dark	−	+	−
No. divisions of chromatin in mature schizont	12–24 Av. 14–16	8–12 Av. 9	8–36† Av. 18

* Deep red; blue, if improperly stained. Presence variable.

† Seen rarely in peripheral blood except in moribund cases.

FLAGELLATES

Trypanosoma.—Two types of trypanosomiasis affecting human patients are recognized: the African type (37) and Chaga's disease (South American trypanosomiasis) (38). A diagnosis of African sleeping sickness can be established only by demonstrating trypanosomes in the blood, lymph nodes, or cerebrospinal fluid. In the acute febrile stages, thick blood films stained by Giemsa's method (p. 34) may reveal the parasite. Another method is to collect 10 ml. of blood and add 1 ml. of a 0.3-per-cent sodium citrate solution. Centrifuge for ten minutes at 1500 r.p.m. Prepare films from the upper sediment and stain by Giemsa's method for thin films. In the early stages of the disease, the parasites may be found in exudate obtained by gland puncture. Make films and stain by Giemsa's method for thin films. In the last stages, obtain cerebrospinal fluid, centrifuge, and stain the sediment by Giemsa's method for thin films. Cultural methods and animal inoculation may be undertaken when the microorganism cannot be demonstrated in films (6b). Animal inoculation may also be followed in the South American type.

In the acute stages of the South American type or Chaga's disease, the parasite may be found in the blood by the same technic as in African trypanosomiasis. However, the microorganism is very fragile and is difficult to demonstrate in the stained film. Recently the complement-fixation test has been recommended (38).

Tissues fixed in Zenker's solution and stained by Giemsa's method (p. 552) will frequently reveal these parasites. In Chaga's disease, they are numerous in the heart muscle.

Leishmania.—Leishmania may incite either a visceral or a cutaneous disease (39, 40). Three species are recognized although morphologically they appear identical: *Leishmania donovani* incites kala azar; *Leishmania tropica*, the cutaneous type of leishmaniasis known as oriental sore; and *Leishmania braziliensis*, the mucocutaneous type of espundia.

The diagnosis of kala azar or visceral leishmaniasis can be established only by demonstrating the parasite in the blood stream or in the reticulo-endothelial cells of the various viscera, particularly liver, spleen, and in the bone marrow (4d). In the acute febrile stages, the parasites may frequently be found in thick blood films or in films prepared from bone marrow, stained by Giemsa's method (p. 34). The parasites appear as endothelial inclusions approximately 1–3μ, oval or round, with a sharply defined outline. The cytoplasm stains light blue and the nucleus, a bright red, while the kinetoplast, a rod-shaped parabasal body and a dot-like blepharoplast, stains a darker red. A concentration method as described for trypanosomes may be helpful. If the parasites are not found in the blood and marrow films, cultural examination (6c) and complement-fixation tests (41) may be undertaken. Chopra (42) and Napier (43) have recommended nonspecific biochemical methods.

In the cutaneous form, the parasites are not found in the peripheral blood. Diagnosis depends upon demonstration of them in the lesion. Aspirate some of the exudate from the base of the lesion as in the collection of material to be examined for *Treponema pallidum* (see p. 335). Spread the material on a glass slide and stain by Giemsa's method in the usual manner for thin films. If parasites are not found, a cultural examination of the material is advisable (6c).

TOXOPLASMA (44–46)

Toxoplasmosis is a protozoan infection incited by *Toxoplasma*. The disease is apparently common in rodents but has only recently been recognized in man. Three types have been reported: (1) congenital encephalitis and hydrocephalus, (2) the acute encephalitis in childhood, and (3) spotted-fever-like disease associated with atypical pneumonia in adults. A diagnosis is established by identification of the protozoan parasite in films made from tissue or fluids stained by the Giemsa method (p. 34), in especially stained tissue sections, by animal inoculation, and by serologic tests.

Toxoplasma is a protozoan the exact classification of which has not yet been determined. It varys in size from 4–7μ in length and 2–4μ in width. In fresh preparations, it is cresent-shaped with rounded ends. The cytoplasm stains blue and the nucleus, a reddish violet. No flagella or outer membranes are present and the absence of a kinetoplast should differentiate it from *Leishmania*. In fixed sections of tissue, the protozoa is only about one-half as large as in stained films. In hematoxylin and phloxine stained sections, the cytoplasm stains pink and the chromatin, dark blue, while with Giemsa's stain, the tinctorial reaction is the same as in the film. Whether the *Toxoplasma* found in man and in animals are the same or different species has not as yet been conclusively proven.

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CHAPTER 33

EXAMINATION FOR PARASITIC WORMS (1–5)

Diseases incited by helminths have been relatively unimportant in the temperate sections of the United States in recent years because of climatic and sociologic factors and improvements in sanitary conditions. Many persons returning to these districts from military service in the tropics may, however, now harbor these parasites. Even though it is anticipated that helminthic infections will not become endemic, the laboratory must be in a position to aid in their diagnosis.

Specimens should be examined promptly after collection. The importance of adequate history and clinical data cannot be overemphasized since the geographic distribution of many parasites is relatively localized. Hence, in addition to pertinent information in regard to clinical manifestations, a knowledge of the places where the patient has been stationed may be particularly helpful in reaching a decision regarding the laboratory procedures that should be undertaken.

Helminths of medical importance include cestodes, nematodes, and trematodes. A diagnosis of helminthiasis depends on finding the adult worm, the larva, or the ovum.

CESTODES

The most important cestodes include *Taenia saginata*, *Taenia solium*, *Diphyllobothrium latum* (6), *Hymenolepis nana*, *Dipylidium caninum*, and *Echinococcus granulosus* (*Taenia granulosa*). A diagnosis of infection with one of these parasites, with the exception of *Echinococcus granulosus*, can usually be made by finding ova or segments in feces.

About 10–15 grams of feces are ordinarily adequate and the specimen is usually satisfactory for several days if a search is to be made only for ova. Examine the gross material with a hand lens. Small worms or proglottides may frequently be found by this procedure.

Place a drop of fecal material about 2 mm. in diameter in the center of a clean slide. Emulsify with a drop of salt solution. Apply a cover glass and examine with the low-power objective.

If ova are not found, prepare films of such a thickness that newsprint viewed through them will appear blurred. After they have dried overnight, cover with balsam, add a cover glass and examine. When the stool is received in glycerol, centrifuge, and wash the sediment in 50-per-cent alcohol before making these preparations.

If the ova are few in number, concentration of the specimen by the zinc-sulfate method is desirable (7). The procedure is essentially similar to that for amebae (see "Examination for Protozoa," p. 482), except that the material is not screened.

In the identification of the various intestinal ova, the charts of Faust (1), Manson-Bahr (4), Stitt (3), Belding (8), and those in other clinical-pathologic texts may prove helpful. The ova of *T. saginata* and *T. solium* resemble each other so closely that identification is difficult. The uterus of *T. saginata* usually has over fifteen main branches while that of *T. solium*, only seven to twelve. Inject one of the gravid proglottides with India ink through a lateral pore and press between two glass slides. Determine whether the uterus corresponds to that of *T. saginata* or *T. solium*.

If a search for the head of a tapeworm is desired, the entire fecal specimen should be submitted in a large container such as a Mason jar. Pass the feces through a 20-mesh wire screen and gently wash under tap water. This procedure separates the worm from the feces. Place the tapeworm carefully in a flat enameled dish with a black bottom. When the narrow neck is reached, examine with a hand lens. The characteristic scolex can then be seen.

A diagnosis of echinococcus infection or hydatid disease (9, 10) can only be established by demonstrating scolices or hooklets in cystic fluid obtained at operation or autopsy, and by the microscopic appearance of the laminated membrane. However, when the disease is suspected, considerable assistance may be obtained from a history of exposure, the presence of a slowly growing tumor, X-ray appearance of the lesion, and the results of complement-fixation (11, 12) and intradermal tests (13).

NEMATODES

Nematodes of medical importance include:

Trichuris trichiura
Enterobius vermicularis
Ascaris lumbricoides
Ancylostoma duodenale
Necator americanus
Strongyloides stercoralis
Trichinella spiralis
Wuchereria bancrofti
Loa loa
Onchocerca volvulus
Dracunculus medinensis

A diagnosis is based on finding the ova, larvae, or worms. In many instances, ova are readily found in feces in the same manner as for cestodes. However, not all worms deposit ova in the feces; the ova of *Enterobius vermicularis* are usually on the skin around the anus (14). A specimen from the perianal skin may be collected with transparent cellulose adhesive tape (15). Pat the surface of the skin with the adhesive surface of the tape; mount it on a glass slide and examine microscopically.

In heavily infested areas, in addition to searching for the ova of *Ascaris lumbricoides* in stools, the bloody sputum of children should be examined for the larvae.

While it is not anticipated that heavy infections of hookworm will occur among the personnel returning from services in the tropics, ancylostomiasis should always be considered, particularly when the patient is found to be anemic.

TRICHINELLA SPIRALIS

Several laboratory procedures are available for the demonstration of *Trichinella spiralis* (16). Examination of the blood is of the greatest importance. Prepare a blood film and examine by Wright's method (p. 42). Eosinophilia is the earliest and most important finding; eosinophils up to 50 per cent have been reported. In severe cases, however, this condition may be absent. In some instances in the early stages of the disease, usually two to four weeks after infection, the larvae may be found in the blood (17). Lake 5 ml. of blood with 10 volumes of 3-per-cent acetic acid. Centrifuge at 1500 r.p.m. for five to ten minutes. Examine the sediment for larvae. A search for adult worms or larvae in the feces is seldom of value. In rare instances, larvae may be found in the sediment of centrifuged cerebrospinal fluid. Precipitin (18, 19), complement-fixation (20), and intradermal tests (19–21) are considered valuable aids to diagnosis, if properly evaluated.

In certain instances, the examination of a biopsy specimen may be advisable. Tissue, approximately 2 by 2 by 0.5 cm., should be taken from a swollen muscle, preferably the deltoid, biceps, or gastrocnemius. Examine a portion histologically by the frozen-section technic (p. 544) and the remainder by a compression method. A special trichinoscope for this purpose has been devised (22). Tease a bit of the muscle in glycerol, place in the trichinoscope and, after compression, examine under the low-power objective.

When the suspected food such as pork, sausage, or bear meat is available, examine first by the compression method and then by the digestion method (23). It may be necessary to feed rats with some of the suspected material. The animals are killed and the parasites sought in the various tissues.

FILARIOIDEA (24–28)

Wuchereria bancrofti, *Loa loa*, and *Onchocerca volvulus* are probably the most important filariae of this group. The early diagnosis of *Wuchereria bancrofti* is important. A history of exposure and the characteristic lymph node enlargement with involvement of the genitalia and lymphangitis are especially significant. Intradermal (25) and complement-fixation tests (27), using antigen from *Dirofilaria immitis* have been recommended as aids in diagnosis. Since the worms may require from twelve to eighteen months to mature, microfilariae may not be found in the blood stream until the patient has had the disease for at least one year. In a search for larvae in the blood, prepare thick films as for malaria parasites, of specimens taken between 10:00 p.m. and 2:00 a.m.; stain by Giemsa's method (p. 34). (Microfilariae can usually be found in the blood stream during the period when the patient sleeps. Hence, if he works at night and normally sleeps during the day, the search should be made during the sleeping period. In certain areas of the South Pacific, the microfilariae have been reported not to have this periodicity in the blood.) They are easily recognized with the low power of the microscope. If the parasites are not found, the concentration method of Knott (29) can be tried.

Lioasis, calabar or eye-worm disease, is prevalent in tropical West Africa. The adult worms migrate in the subcutaneous tissue of man. The microfilariae may be found in the peripheral blood or in fluid aspirated from the calabar (intermittent) swellings. Since they have been reported to be more numerous in the blood during the day than at night, specimens should be collected between 10:00 a.m. and 2:00 p.m. and examined as for *Wuchereria bancrofti.*

Onchocerciasis (30) is prevalent in parts of Gautemala, Mexico, and certain sections of Africa. Usually the disease is characterized by small skin tumors, particularly of the scalp. The diagnosis can be made early in the disease by demonstrating microfilariae in either stained or unstained films prepared from aspirated material from the tumors. A biopsy specimen may also be examined histologically in the usual manner.

TREMATODES

The most common trematodes include *Schistosoma* (31–35), *Fasciolopsis buski*, *Clonorchis sinensis* (36), and *Paragonimus westermani* (37, 38). Schistosomae are probably the most important from the practical point of view. *Schistosoma mansoni* and *Schistosoma japonicum* incite liver and intestinal infections, respectively. In the case of *Schistosoma hematobium*, the infection is usually in the urinary tract although occasionally the liver is involved. Ova of *S. mansoni* and *S. japonicum* can be found in feces. Mix about 15 grams of feces with lukewarm water and dilute until it is the consistency of thin emulsion. Pour in a 16-oz. sedimentation glass and allow to settle. Decant the supernatant and refill with water. Repeat four times. Decant the supernatant. Transfer the sediment to a glass slide and examine microscopically for ova. A drop of D'Antoni's iodine (p. 32) is helpful. Material from ulcers obtained by protoscopic examination (31) will frequently reveal the characteristic ova. An acid-ether method for demonstrating the ova has recently been recommended (35).

If infection is indicated and ova are not found, a small portion of the fecal material can be placed in a flask of water and kept in the light for from twelve to twenty-four hours. After the miracidia leave the eggs, they can be seen as actively swimming organisms. They are ciliated and appear somewhat larger than the ova from which they hatched.

Serologic and skin tests may also be helpful in making an early diagnosis (35).

Schistosoma hematobium can be found in urine. Collect a specimen in a sedimentation glass. Allow the eggs, pus, and blood to settle. Pipette the sediment from the bottom on a glass slide and examine microscopically. If ulceration of the bladder or urethral mucosa is present, the lesion may be scraped and the material examined for ova.

The most important intestinal and liver flukes are *Fasciolopsis buski* and *Clonorchis sinensis*, respectively. Infections with these trematodes can be diagnosed by demonstrating the ova in feces. For detailed study of their appearance, consult a text containing illustrations such as that of Faust (1), Manson-Bahr (4), and Stitt (3).

Infection with *Paragonimus westermani*, the lung fluke, occurs in the Far East, chiefly Korea. Persons with chronic cough and bloody sputum may be infected with this parasite. Hence, in addition to examination for evidence of tuberculosis, a drop of bloody sputum can be examined microscopically for the ova of this fluke. Concentration of sputum is helpful.

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CHAPTER 34

EXAMINATION FOR EVIDENCE OF INFECTIOUS MONONUCLEOSIS

The clinical manifestations of infectious mononucleosis vary markedly and often suggest unrelated diseases; the epidemic form, which usually occurs among children, is commonly referred to as glandular fever (1). Although the etiology has not been established, the agglutination test with sheep red blood cells is a distinct aid in diagnosis (2–4). Injections of horse serum, particularly when serum sickness occurs, and administration of bacterial vaccines or other types of foreign protein may also, however, give rise to agglutinative properties (5, 6). The significance of the reaction may be determined by absorption tests with guinea pig kidney tissue and beef erythrocytes (7, 8).

BLOOD

Serologic Examination

Inactivate a 1:10 dilution of serum in salt solution by heating in a water bath for fifteen minutes at 55°–56°C. Prepare from this a series of dilutions in geometric progression. Combine equal volumes of serum dilutions and of a 1-per-cent suspension of washed sheep red blood cells collected from twenty-four to forty-eight hours previously. For control, combine equal volumes of the suspension of sheep cells and series of dilutions of two sera, one known to agglutinate and the other not to agglutinate these cells. Shake thoroughly, incubate for four hours at approximately 37°C., and read the reactions immediately.

Recording Results.—Record the degree of agglutination as follows:

4+ complete agglutination, when the cells form a plaque that is not broken by shaking the tube
3+ definite agglutination, when the cells form large clumps but not a single plaque
2+ partial agglutination, when the clumps are small
+ questionable reaction
− no reaction

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CHAPTER 35

EXAMINATION OF BLOOD

Hematologic examinations provide important aid in clinical diagnosis (1–4). A routine examination should usually include hemoglobin determination, red cell count, white cell count, and differential count. Frequently, it is necessary also to make a platelet count, to determine coagulation time, make sedimentation test, reticulocyte count, determine fragility of red cells, and, in special instances, study sternal marrow films. Determination of blood group including the Rh factor and matching also form an integral part of blood examinations. In purpuric and hemorrhagic states, it is important to determine platelet count, bleeding time, clot retraction time, clot friability, and prothrombin.

COLLECTION OF SPECIMENS

Collect the blood from the lobe of the ear when only a few drops are required. The clothing should be protected with a towel. Clean the surface of the ear lobe with alcohol and gently massage. Allow the alcohol to dry. Puncture the tissues with a sharp lancet deeply enough to obtain a large drop of blood. Do not squeeze the tissues after the puncture has been made. When a larger specimen is needed, obtain blood from a vein, preferably from the median basilic vein.

DETERMINATION OF HEMOGLOBIN

Colorimetric Method

Newcomer's Method.—With the special pipette provided with the Duboscq-Klett colorimeter, draw up blood to the 25-cmm. mark and discharge into a test tube containing 10 ml. of 0.1 *N* hydrochloric acid. After mixing, allow the tube and contents to stand for at least thirty minutes. Insert the brown-glass standard supplied for the instrument in the top of the left-hand plunger of the colorimeter and fill the cup underneath to a height of 10 mm. with distilled water. Adjust the graduated scale to 10 before reading. The right-hand cup is then filled with the acid-hematin mixture, and matched against the standard. Read the height at which both match. Use the table supplied by the manufacturer to obtain grams per 100 ml. and per cent hemoglobin. With this instrument, 15.3 grams of hemoglobin per 100 ml. of blood represent 100 per cent.

Hellige's Modification of Sahli's Method.—Place about 5 drops of 0.1 *N* hydrochloric acid into the special graduated tube provided with the Sahli-

Hellige hemometer. Draw blood up to the 20-cmm. mark into the special pipette. Note the exact time. Gently blow out the blood into the hydrochloric acid solution and wash the pipette three times by sucking a little water into it. Add the washings to the hydrochloric acid solution. Shake the contents of the tube until the blood is well mixed with the hydrochloric acid and the solution shows a uniform dark-brown appearance. Add water a drop at a time while stirring the mixture with the glass rod supplied, until the solution in the graduated tube matches the standard. Read the result. The procedure should not occupy more than five minutes.

With this instrument, 14.5 grams of hemoglobin per 100 ml. of blood represent 100 per cent.

Photoelectric Method

Directions for cleaning and standardization of the colorimeter are given on p. 534. As the standard, use the sealed acid hematin tube supplied with the Klett-Summerson colorimeter. The solution is usually stable for at least six months if kept in the refrigerator. Check occasionally for possible deterioration by making hemoglobin determinations and comparing the findings with an oxygen-capacity or carbon-monoxide-capacity determination. (See "Gasometric Chemical Analysis and Related Technics," p. 109.)

To make a hemoglobin determination, place 5 ml. of 0.1 *N* hydrochloric acid in a test tube. Collect 0.02 ml. of blood in a pipette calibrated "to contain." Remove excess blood from the outside of the pipette and then deliver the blood into the tube containing hydrochloric acid solution. Rinse the pipette by repeatedly drawing up and expelling portions of the liquid in the tube, mixing the contents well with the tip of the pipette at the same time. Remove the pipette. Allow this acid hematin mixture to stand at room temperature for at least thirty minutes, to permit maximum color development.

Fill a colorimeter tube with distilled water and place it in the Klett-Summerson colorimeter. Use green filter No. 54. Adjust the instrument to the 0 reading. Remove the tube. Mix the acid hematin standard by gently inverting the tube several times in order to disperse the particles evenly through the liquid. Place in the colorimeter. Turn the scale until the pointer comes back to its zero position, then read.

Rinse the colorimeter tube containing distilled water used for the zero reading with a little of the unknown acid hematin solution and use the rest for the colorimetric reading.

Calculate the grams of hemoglobin per 100 ml. of blood from the colorimeter readings of the known and unknown solutions. (Grams of known as stated on the tube): (reading of known) = (grams of unknown):

(reading of unknown.) The following method may also be used. Let S be the concentration of hemoglobin in grams per 100 ml. in the standard and X be the concentration of the unknown solution, and let R_S be the colorimetric reading of the standard, and R_x be that of the unknown; then $S:R_S = X:R_x$ whence $X = S \cdot R_x/R_S$.

If it is desired to convert grams of hemoglobin to per cent, calculate in accordance with the method given by Van Slyke (5) or in other standard tests.

Phillips and others (6) have developed a method of obtaining specific gravities of whole blood and plasma using copper sulfate solution of known specific gravity. From these findings, by the use of line charts, the hemoglobin can be estimated. The method is very useful, especially when large numbers of determinations are to be made and where attainment of minimum requirements of hemoglobin concentration is to be tested.

BLOOD COUNT

Use Levy counting chambers with improved Neubauer ruling, pipettes, and special cover glasses, all of which have been tested by the United States Bureau of Standards.

Be sure that all of the apparatus is dry and free from dust or lint. Clean pipettes immediately after use by rinsing several times with water followed by alcohol and then by ether. Wash the cover glasses and counting chambers in water and polish with lens paper.

Include all the cells or platelets that touch the lower and right-hand sides but not the upper and left-hand sides of the squares being counted.

Erythrocyte Count.—Hold a pipette designed for this purpose horizontally and draw blood up to the 0.5-mark. Wipe the blood from the outside of the tip and quickly insert the tip into Hayem's solution (p. 36) or Gowers's solution (p. 35). Draw up this mixture to the 101-mark to give a dilution of 1:200. Close the tip with the thumb and place the index finger over the kinked rubber at the end of the tube. Gently shake the pipette for two to three minutes to insure proper mixing. The pipette should not be shaken in the direction of the longitudinal axis or held in a vertical position. Pull off the tubing and, with the index finger, control the flow from the pipette. Discard the first two drops. Then place the cover glass over the chamber and fill it by touching the tip of the pipette to the edge of the cover glass. The size of the drop should be such that it covers the ruled area. Allow two to three minutes for the cells to settle. With the high-dry objective of the microscope, count the cells in eighty small squares. Multiply this figure by 10,000 to give the number of erythrocytes per cubic millimeter.

Leucocyte Count.—Hold a pipette designed for this purpose horizontally and draw blood up to the 0.5-mark. Wipe the blood from the outside of the tip and draw up acetic acid crystal violet solution (p. 30) to the 11-mark to give a 1:20 dilution. Shake.

Fill the counting chamber as for a red cell count and allow the cells to settle for three minutes. With the low-power objective, count the cells in the middle and four large corner squares (each 1 sq. mm.). Multiply by 40 the number of cells counted in the five large squares to determine the number of leucocytes per cubic millimeter.

Platelet Count.—Speed is essential. Use a pipette designed for counting erythrocytes. Holding it in a horizontal position, draw Rees and Ecker dilution fluid (p. 39) to the 0.5-mark and blood until the fluid reaches the 1-mark. Then draw up the diluting fluid until the mixture reaches the 101-mark, thus obtaining a 1:200 dilution. Shake the pipette several minutes; discard the first 3–4 drops; and then fill the counting chamber in the usual manner. Allow the platelets to settle for twenty minutes and keep the chamber covered with the top of a Petri plate to prevent evaporation. Platelets appear as highly refractile minute lilac bodies. With the high-dry objective, count them in the 100 smallest squares. Multiply by 8,000 to determine the number of platelets per cubic millimeter. Approximately 230,000–400,000 platelets are usually found per cubic millimeter of blood in normal individuals.

PREPARATION AND EXAMINATION OF BLOOD FILMS

Use clean slides that have been stored in 95-per-cent alcohol. Remove them from the alcohol and flame just prior to use. Obtain blood in the usual way. When a drop collects, touch it with a slide and spread at once with the edge of another slide. The thickness of the film can be regulated by the angle employed. An angle of 35° is satisfactory for thin films. Dry the films in the air and do not fix with heating; the staining solutions act as fixatives.

If preferred, films may be prepared on cover glasses by collecting the drop of blood on one, placing another over it, and immediately drawing them apart. After the films have been stained, they can be inverted over a drop of immersion oil on a clean slide or, if a permanent mount is desired, neutral xylol-dammar (p. 548), can be used.

Wright's stain (p. 42) has been found generally useful for studying blood films although Giemsa's (p. 34) may be preferred when an examination for parasites is made. It is best when searching for spirochetes to overstain.

Differential Leucocyte Count.—Examine the stained film with an oil-immersion objective using a mechanical stage. In a preparation properly stained by Wright's or Giemsa's method, the red blood cells are an orange or pink color, the nuclei of the neutrophile, dark blue; neutrophilic granules, a reddish lilac; eosinophilic granules, red; platelets, violet blue; cytoplasm of the lymphocytes, light blue. Myelocytes have dark blue or purplish nuclei and various types of granules; myeloblasts, blue nuclei and nucleoli, no granules. Monocytes stain very much like lymphocytes and may contain a few red granules.

Record abnormalities in the size, shape, and color of the red cells, and evidence of polychromatophilic or basophilic stippling. Search for various types of nucleated red blood cells, particularly megaloblasts and record the number found during the counting of 300 leucocytes. Search for the presence of parasites or inclusions. Also, make an approximate estimate of the number of platelets per field and record their distribution in clumps or otherwise. Consult standard texts for a detailed description of methods employed.

Count 300 leucocytes and record the number of each type.

Observe the neutrophiles for abnormalities such as toxic granules. If myelocytes, myeloblasts, lymphoblasts, plasma cells and degenerated forms are found, determine the number of each. For the appearance of abnormal cells, study charts in texts on hematology.

Farley, St. Clair, and Reisenger (7) have designated polymorphonuclear leucocytes in which the lobes of the nucleus are connected by a fine strand of nuclear material as filamentous neutrophiles and all others as nonfilamentous neutrophiles. The average normal nonfilamentous neutrophile count is 8 per cent; the upper limit, 16 per cent. The percentage of nonfilamentous neutrophiles present may be of material diagnostic value. This may be obtained by counting 300 leucocytes and determining the percentage of nonfilamentous neutrophiles.

The granulocytic type of leucocyte can be distinguished by means of the peroxidase reaction. By the method of Sato and Sekiya (p. 39) erythrocytes stain pink; nuclei and cytoplasm of lymphocytes, red. Granulocytes, with the exception of myeloblasts, contain blue-black granules or red basophilic granules.

When Washburn's method (p. 41) is used, polymorphonuclear neutrophiles and myelocytes have large black granules; the eosinophilic granules are refractile, with a dark periphery. Basophiles have black granules at the periphery of the cells. Lymphocytes and early stem cells show no granules; monocytes, a few clumped granules.

Staining of Reticulocytes.—Place a drop of blood on one end of a clean slide. With a glass rod, deposit a drop of brilliant cresyl blue (p. 32) stain beside it. Wipe off the excess stain from the rod and mix the two drops thoroughly. Make a thin blood film in the usual manner and stain with Wright's stain. The reticulocytes are spotted with irregular blue areas. Count at least 1,000 red blood cells and record the number of reticulocytes. Report the percentage found. Determination of the mean cell diameter is important but may require special apparatus (8, 9).

COAGULATION TIME

Lee-White Method (10).—Collect about 3.5 ml. of blood from a vein in a 5-ml. syringe in which salt solution has been drawn up and expelled two or three times. Expel 1 ml. of blood into each of three clean and absolutely dry test tubes of about 8 mm. in diameter. Tilt each tube gently at 1-minute intervals until a firm clot has formed so that the tube can be inverted. The average time required for each of the three tubes to clot represents the clotting time of the blood, which for the normal individual averages between five and ten minutes.

Capillary Tube Method.—Use fine capillary tubes, 1–2 mm. in diameter and about 125–150 mm. in length. Fill four tubes with blood. At 30-second intervals, break about 25 mm. off the end of each tube until a fibrin strand is found to connect the pieces. The time required to do this represents the coagulation time which, for the normal individual with this method is generally three to eight minutes.

SEDIMENTATION TESTS

Determination of the sedimentation rate of erythrocytes may furnish data of considerable diagnostic value (11).

Cutler's Method (12, 13).—Use 5-ml. Cutler tubes for the purpose. Aspirate 0.5 ml. of a 3-per-cent sodium citrate solution into a syringe. Then puncture a vein and draw the blood up to the 5-ml. mark. Withdraw the needle from the vein, draw back the piston of the syringe about 1 cm. and gently tilt the syringe back and forth to insure uniform mixing of the blood and citrate. Deposit the citrated blood in the Cutler tube. Stopper with a paraffined cork and place upright in a rack. Before reading, invert the tube two or three times to insure an even distribution of red cells.

Record the readings at 5-minute intervals for one hour on Cutler's graphic charts. The horizontal lines represent the divisions of the sedimentation tube and the vertical, time intervals. This gives data for a graph which shows the sedimenting column of red cells at any period of time during the hour. The sedimentation index is the total sedimentation of the red cells for one hour expressed in millimeters. For men, this is normally 2–8 mm.; for women, 5–6 mm., but during menstruation, it may be as high as 12.

Wintrobe-Landsberg Method (14).—Use the Wintrobe hematocrit. Collect 5 ml. of blood in a tube containing a mixture of 6 mg. ammonium oxalate and 4 mg. potassium oxalate as an anticoagulant. After thoroughly mixing with the blood, withdraw into the special capillary pipette supplied. Pass the pipette to the bottom of the hematocrit and expel the blood. As pressure is applied to the bulb, the blood is slowly forced out and the pipette is withdrawn. Fill the hematocrit to the 0 mark. Allow to stand vertically for one hour, then determine the sedimentation rate. After centrifugation at 3000 r.p.m. for thirty minutes, determine the volume of packed red cells by reading directly from the numbers on the right side of the scale. Wintrobe gives charts for use in correcting the sedimentation rate for various anemias which may be helpful in interpreting the finding obtained.

HEMORRHAGIC DISEASES

The icterus index, the fragility of red blood cells, and a determination of the bilirubin content of the blood may furnish data of value in the diagnosis of hemolytic diseases.

Icterus Index (15).—Obtain by venous puncture 5–10 ml. of blood. Be sure that no hemolysis occurs. Centrifuge immediately. Place 1 gram of potassium dichromate in a 100-ml. volumetric flask and dissolve in approximately 50 ml. of distilled water. Add 0.1 ml. of concentrated sulfuric acid. Then add distilled water to make 100 ml. Place 1 ml. of this stock solution in a 100-ml. volumetric flask and add distilled water to make 100 ml. This dilution represents one unit in the icterus index test and is used as the standard. Place 1 ml. of the standard potassium dichromate solution in the left-hand cup of a Duboscq-Klett colorimeter. Adjust the colorimeter to 15. Place 1 ml. of the serum in the right-hand cup or, if the color of the serum is deeper than that of the standard, dilute 1 ml. with salt solution until it is just lighter than the standard, then add 1 ml. to the cup. Match the color against the standard. Use the following formula:

$$\frac{15}{\text{Reading}} \times \text{dilution of serum} = \text{icterus index.}$$ The normal is 3–5.

Fragility Test.—To prepare a 0.75-per-cent solution of sodium chloride, dry 1 gram of sodium chloride, C.P., over sulfuric acid in a desiccator. Weigh exactly 0.75 grams and dissolve in a 100-ml. volumetric flask adding distilled water to make 100 ml. Set up eighteen test tubes (100 by 14.5–16 mm.) in a rack and number from left to right. In the first tube, measure 4.8 ml. of the 0.75-per-cent sodium chloride solution; in the second, 4.6 ml.; in the third, 4.4 ml., continuing until all the tubes have been filled. Add sufficient distilled water to each tube to bring the volume to 5 ml. Collect blood in the usual manner and mix well. Measure 0.2 ml. into each tube.

Set up a normal control. Place the racks in the refrigerator for three hours and read. Observe in which tube hemolysis first begins.

Hemolysis normally begins in the tube containing 0.45 per cent of sodium chloride and is complete in the one containing 0.33 per cent. Abnormal fragility is present when hemolysis begins at a concentration higher than 0.45 per cent salt solution and is complete at a greater concentration than 0.33 per cent.

Determination of Bilirubin (16).—Prepare a standard bilirubin solution as follows: Dissolve 2 mg. of bilirubin in 2 ml. of water and add 1 drop of 10-per-cent sodium hydroxide. Dilute with water to make 20 ml. and add 10 ml. of freshly prepared diazo reagent (p. 32), 20 ml. of saturated ammonium sulfate solution, and 80 ml. of 95-per-cent ethyl alcohol. When a red color has developed, add 20 ml. of concentrated hydrochloric acid; let stand for four hours, and store in the refrigerator. This solution will keep for three months.

To 2 ml. of plasma, add 1 ml. of freshly prepared diazo reagent (p. 32) and mix. Then add 2 ml. of saturated ammonium sulfate solution and 8 ml. of 95-per-cent ethyl alcohol. After a red color has developed, add 2 ml. of concentrated hydrochloric acid, mix, and let stand for ten minutes. Filter. Place the filtrate in the colorimeter cup and set the cup at 10 mm. Match the standard bilirubin solution against the unknown. To calculate the amount of bilirubin:

$$\frac{\text{reading of standard}}{\text{reading of unknown}} \times 10 = \text{mg. of bilirubin per 100 ml. of plasma.}$$

Normal blood plasma contains about 0.1–0.5 mg. of bilirubin per 100 ml.

BLOOD GROUPING (17–20)

Blood grouping and matching should be undertaken only in laboratories that are fully equipped and have personnel trained for this type of work.

The Landsteiner classification of O, A, B, AB is used. High titer sera of groups A and B must be available. The sera should be free from pseudoagglutinative properties. In addition to the two agglutinogens, A and B, there are several subgroups that must be considered. These are very important, especially when repeated transfusions are required.

Allow a large drop of blood collected in the usual manner to fall into a tube 10–11 mm. in diameter and containing 1 ml. of salt solution. This will give approximately a 5-per-cent cell suspension. Transfer with a capillary pipette or Asepto syringe 1 drop of this suspension to each of three tubes 10–11 mm. in diameter. Place 1 drop of type-A serum in the first tube and 1 drop of type-B serum in the second. Add 1 drop of salt solution to each of these and 2 drops to the cells in the third tube which is a control. Centrifuge the tubes at 2000 r.p.m. for three minutes. Place them in a rack and shake until the cells in the control tube are evenly suspended. The presence of clumps or solid clots indicates agglutination. By reference to table 62 the group to which the patient's blood belongs can be determined.

Have cells of known groups A and B available to include as controls during the test.

TABLE 62

Relationships of the four blood groups

CELLS OF GROUPS	SERA OF GROUPS			
	O	A	B	AB
O	−	−	−	−
A	+	−	+	−
B	+	+	−	−
AB	+	+	+	−

− = no agglutination.
+ = agglutination.

BLOOD MATCHING TESTS

Before a transfusion is made, the blood of the donor and recipient must be shown to be compatible.

Collect a few drops of blood from the prospective recipient and allow to clot so that the serum can be separated. Also collect two large drops of blood in 2 ml. of salt solution. Collect similar specimens from the prospective donor. Place 2 drops of the recipient's serum in a small test tube, add 2 drops of a 5-per-cent suspension of the cells from the donor, and 2 drops of salt solution. In the second test tube, place 2 drops of serum from the donor, add 2 drops of a 5-per-cent suspension of cells from the recipient, and 2 drops of salt solution. Centrifuge at 1000 r.p.m. for one minute, then gently shake the tubes to resuspend the cells. If the blood of the donor and recipient are compatible, there should be no agglutination or clumping of cells in any of the tubes. The findings can be confirmed by microscopic examination.

Rh Factor.—The blood of about 85 per cent of individuals has been found to contain an agglutinogen that has been designated as Rh by Landsteiner and Wiener (21). The remaining 15 per cent lack this agglutinogen and their blood apparently forms an agglutinin against it after blood containing the Rh factor has been introduced into the circulation. In pregnancy, if the mother is Rh negative and the fetus Rh positive, the mother may become immunized and a subsequent transfusion with Rh positive blood may lead to a serious or fatal hemolytic reaction. Similar accidents may follow repeated use of Rh positive blood for transfusions in an Rh negative recipient (22). The test for the Rh factor should be made in all cases of pregnancy and when patients are receiving repeated transfusions. Also, when transfusions are given to infants affected with erythroblastosis fetalis, blood from group O, an Rh negative donor, should be used if possible. The technic and data in regard to interpretation of results will be found in the publications of Landsteiner and Wiener and others (21–25).

CULTURAL EXAMINATION

See "General Instructions," p. 263.

SEROLOGIC TESTS

See "General Instructions," p. 264; "Serologic Tests for Evidence of Syphilis, Tuberculosis, and Gonococcus Infection," p. 361.

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CHAPTER 36

EXAMINATION OF URINE

Uranalysis is best undertaken in a hospital laboratory or in the physician's office where the specimen can be promptly examined. Various tests not mentioned here may need to be performed. For details, standard texts on clinical pathology should be consulted.

COLLECTION OF SPECIMEN

Urine should be examined as soon after being voided as possible. Bacillary incitants of enteric disease usually remain viable for a few days if the specimen is combined with 30-per-cent glycerol. When bacteriologic examination is required, the use of aseptic precautions is necessary. For qualitative tests, a single specimen may be satisfactory; for quantitative tests, a representative sample of a 24-hour specimen should be collected.

MACROSCOPIC EXAMINATION

Record the quantity received and describe the color, appearance, and odor. Determine the reaction to litmus paper and the specific gravity by a urinometer, which must float freely in the fluid. Remove any bubbles or debris from the surface of the urine with a piece of dry filter paper. Always read from the bottom of the meniscus.

MICROSCOPIC EXAMINATION

Moist Preparations

Centrifuge the urine from three to five minutes at 1000 r.p.m. Remove the supernatant fluid, transfer a small amount of the sediment to a slide by means of a capillary pipette, and apply a cover glass. Examine several such preparations made separately from the upper and lower portions of the sediment when it is abundant. Before a cover glass is added, an examination with low magnification for casts may be desirable. Be sure that the field is not illuminated too brightly. In acid urine, amorphous urates, uric acid crystals, calcium oxalate, and hippuric acid are found; in alkaline urine, both amorphous and triple phosphates as well as ammonium urates are usually present. Make a careful search for pus cells, red blood cells, tissue cells, and casts. Record the relative number of bacteria; for example, many, moderate number, or few. It has become necessary to recognize crystals of the sulfonamides in the urine when these drugs have been used in treatment (1).

BACTERIOLOGIC EXAMINATION

When a request is made for an examination for tubercle bacilli or other bacteria, isolate and identify the microorganisms present as outlined in chapters dealing with the individual species concerned.

CHEMICAL EXAMINATION

Albumin

QUALITATIVE TESTS

Filter the specimen until it is clear.

Purdy's Heat Test.—This test is used as a routine procedure. To 15–20 ml. of urine in a test tube, add approximately 3 ml. of a saturated solution of sodium chloride and 8 drops of a 50-per-cent solution of acetic acid. Mix by inverting the tube, and cautiously boil the upper portion of the fluid. A white cloud in the heated area denotes the presence of albumin. Bence-Jones protein produces a white cloud that disappears upon boiling and reappears upon cooling.

Heller's Ring Test.—Pour 2 ml. of nitric acid into a test tube, and cautiously layer the same amount of urine over the acid by allowing it to run slowly down the side of the tube. A white ring at the zone of contact of the two fluids indicates the presence of albumin. Whenever the tests show more than a faint trace of albumin, estimate the amount by Esbach's method.

Sulfosalicylic Acid Test (2).—This test is of considerable value and is preferred by many.

QUANTITATIVE TEST

Esbach's Method.—Filter the urine and determine the reaction with litmus paper. Add acetic acid, if necessary, to give an acid reaction. If the qualitative test has indicated a large amount of albumin, dilute the urine with water. Pour into an Esbach tube to the U-mark and bring the volume to the R-mark by the addition of Esbach's reagent (p. 33). Insert a rubber stopper, mix the contents by inverting the tube slowly several times, and then place in the cold room. After twenty-four hours, the height of the precipitate, as determined by graduations on the tube, indicates the amount of albumin in grams per liter. Divide by ten to obtain the percentage, and, if the urine was diluted, multiply by the dilution factor.

Sugar

QUALITATIVE TESTS

Benedict's Test.—If the specimen has been preserved with chloroform, boil it, or if albumin in more than a trace has been found, boil, acidify, and filter. To 5 ml. of Benedict's solution (p. 31) in a test tube, add 8 drops of urine. Heat for five minutes in a bath of boiling water, then allow to cool slowly. The formation of a brick-red, yellow, or green precipitate demonstrates the presence of sugar. The reading is not made until the mixture is cool, since precipitates due to very small amounts of sugar may not become visible until then.

When sugar is present, examine the specimen also for acetone.

Phenylhydrazine Test.—If the urine is not clear, to about 10 ml., add approximately 1 gram of pure blood charcoal, heat with occasional shaking, and boil for about five minutes. Filter. Thoroughly mix in a mortar 2 parts of phenylhydrazine hydrochloride and 3 parts of sodium acetate by weight. Place approximately 0.5 gram of this mixture in a test tube, 12–14 mm. in diameter, and add about 5 ml. of urine and an equal amount of distilled water. Shake well. Heat on a boiling water bath for approximately three-quarters of an hour. Allow to cool as slowly as possible and preferably leave overnight. Examine the sediment with a low-power dry objective.

If time is a factor, the examination can be made by using a Pyrex test tube, about 25 mm. in diameter, and boiling over a free flame. A glass bead or section of glass tubing may be required to prevent bumping. The mixture should be concentrated to a volume of approximately 2 or 3 ml. It can then be poured into a small test tube which has been previously warmed and allowed to cool at room temperature.

Crystals of dextrosazone (glucosazone) are yellowish in color, needle-shaped, and usually grouped in clusters.

QUANTITATIVE TEST

Benedict's Macro Test.—Place 25 ml. of Benedict's solution for quantitative estimation of sugar (p. 31) in a porcelain evaporating dish with 10 grams of sodium carbonate, anhydrous, and a few small pieces of pumice stone. Bring the mixture to the boiling point and keep it boiling vigorously throughout the titration. Add the urine rapidly from a graduated burette. When the sugar content is high (more than a slight trace), dilute 1:10. In the presence of sugar, a white precipitate forms and the blue color of the solution fades. When the color becomes pale, add the urine more slowly and then drop by drop until the end-point is reached; that is, the disappearance of the blue color. Read the amount of urine used from the burette and calculate the percentage as follows:

Let X equal the quantity in milliliters of undiluted urine used. Then

$$\frac{0.05}{X} \times 100 = \text{glucose concentration in grams per 100 ml.}$$

(0.05 gram of glucose is required to reduce 25 ml. of the reagent.)

Bile

Shake the urine in a bottle or test tube. If bile pigments are present, they impart a yellowish or brownish color to the foam.

Blood

Tests for blood in urine are of value only when the possibility of extraneous blood (e.g., menstrual) is eliminated. Blood may be detected by the benzidine test. Mix equal quantities of urine and benzidine reagent (p. 31). A blue color appears in the presence of blood.

Acetone

Lange's Test.—To 5 ml. of urine in a test tube, add 5 drops of glacial acetic acid and 5 drops of a saturated aqueous solution of sodium nitroprusside. Tilt the tube and layer with ammonium hydroxide (26–28 per cent NH_3 by weight). A purple color at the zone of contact denotes the presence of acetone.

Rothera's Test.—To 5–10 ml. of urine in a test tube, add 1 gram of ammonium sulfate and 2 or 3 drops of a fresh concentrated solution of sodium nitroprusside. Tilt the tube and layer with ammonium hydroxide (26–28 per cent NH_3 by weight). A permanganate-colored ring indicates the presence of acetone.

Diacetic Acid

Gerhardt's Test.—To 5–10 ml. of urine in a test tube add, drop by drop, a 10-per-cent solution of ferric chloride until the precipitation of the phosphates is complete. Then filter and add a small quantity of the ferric-chloride solution to the filtrate. If diacetic acid is present, a Bordeaux red color is formed which disappears upon boiling. Several minutes' boiling is required; simply bringing the fluid to the boiling point will not suffice. The test may also be performed by the ring method, which results in a more intense coloration.

If desired, a few drops of a 35- to 40-per-cent solution of ferric chloride may be used in place of the 10-per-cent solution. This will eliminate the necessity for filtering.

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(2) Exton, W. G. A simple and rapid quantitative test for albumin in urine. J. Lab. and Clin. Med., 1925, **10,** 722–735.

CHAPTER 37

EXAMINATION OF CEREBROSPINAL FLUID

When the patient has symptoms of meningitis, a specimen of cerebrospinal fluid should be sent in the miscellaneous tube outfit (see "Appendix C," p. 917). When an examination for evidence of neurosyphilis or a disease of the central nervous system other than meningitis incited by bacteria is desired, the outfit containing two tubes, one with a conical base for the cerebrospinal fluid and one of the usual type with needle for the blood specimen should be used (see "Appendix C," p. 913). It is important that a specimen of the patient's blood accompany the cerebrospinal fluid.

COLLECTION OF CEREBROSPINAL FLUID

The source of the fluid, whether collected by lumbar, cisternal, or ventricular puncture must be known. For most purposes, particularly in syphilitic cases, lumbar puncture is the method of choice. When the fluid is collected in this manner, two sharp needles that have been thoroughly cleaned and sterilized preferably in dry heat should be available. The needle is usually inserted in the fourth lumbar interspace. If the fluid contains blood, the needle should be removed and another tap made in the next higher interspace with the second needle, since even slight contamination with blood may render it unsatisfactory for examination. When, in spite of these precautions, blood is present, the blood cells should not be removed before the specimen is sent to the laboratory. The presence of blood plasma in the fluid may render the laboratory findings unreliable unless the relative amount of this contamination can be estimated on the basis of the number of blood cells that enter the cerebrospinal fluid.

CONDITION OF SPECIMEN

Review the accompanying data, inspect the cerebrospinal fluid, and record the amount received, opacity, color, presence of blood, particles of debris, and coagulum. Have specimens that are purulent, turbid, or that contain a fibrin web, as well as any with a history suggestive of meningitis examined bacteriologically. On all other specimens have a cell count made, a complement-fixation test for syphilis performed, total protein determined, and a colloidal gold test made. If these tests are made, the determination of the sugar content will furnish little, if any, information of additional diagnostic value. Hence, the routine performance of such tests is not necessary.

Contamination with Blood.—The approximate admixture of hemoglobin can be determined by comparison with a series of dilutions of a standardized solution of hemoglobin. This solution may be made by direct dilution of freshly collected human blood in distilled water, or from a suitably adjusted solution of citrated sheep blood. Measure 1 ml. of citrated sheep blood with a volumetric pipette into a 25-ml. volumetric flask. Bring to 25 ml. by the addition of 0.1 *M* hydrochloric acid. Mix thoroughly with a clean pipette and allow to stand thirty minutes for maximum color change. With a volumetric pipette, transfer 10 ml. of the mixture to a clean 50-ml. volumetric flask and bring to 50 ml. by the addition of 0.1 *M* hydrochloric acid. Mix thoroughly with a clean pipette and determine colorimetrically the per cent of hemoglobin.

In a Klett-Summerson photoelectric colorimeter, (green filter #54), compare the deviation caused by the preparation of citrated sheep blood with that caused by an acid hematin standard of known hemoglobin content. To determine the hemoglobin content of the sheep blood, multiply the reading obtained with it by the hemoglobin content of the acid hematin standard and divide the product by the reading obtained with the standard. (See also "Examination of Blood," p. 503.)

Assume that 15 mg. per 100 ml. represents the hemoglobin content of normal human blood. Lake citrated sheep blood in a quantity of distilled water sufficient to produce the equivalent of 1:100 solution of normal human blood. For example, if the sheep blood contains 12.4 mg. per 100 ml., the hemoglobin content is approximately 81 per cent of that of normal blood. Make allowance for the use of citrate solution. Thus 2 ml. of citrated blood of this hemoglobin content plus 79 ml. of distilled water will give the equivalent of a 1:100 solution of normal human blood. Dilute this standardized solution 1:10, 1:20, 1:30, 1:40, 1:60, 1:80, and 1:100 to make standards equivalent to 1:1000, 1:2000, 1:3000, 1:4000, 1:6000, 1:8000, and 1:10,000 dilutions of blood. Stopper in 100- by 4.5-mm. tubes and store in the refrigerator when not in use. Prepare a new series each week, using 1-per-cent formalin as diluent.

Against a white background, compare the supernatant cerebrospinal fluid with this standard, and record the color. Preparations more concentrated than 1:1000 may be made but are not necessary, because blood contamination greater than 1:1000 usually renders the cerebrospinal fluid entirely unsatisfactory for examination. The amount of protein attributed to blood admixture represented by the color standard is as follows:

1:1000—20 mg. per 100 ml.; 1:2000—10 mg. per 100 ml.;
1:3000— 7 mg. per 100 ml.; 1:4000—5 mg. per 100 ml.;
1:6000— 4 mg. per 100 ml.; 1:8000 and 1:10,000, negligible.

Report fluids that have a color corresponding to 1:1000 or greater concentration of blood as yellow (or red), those corresponding to 1:2000 and 1:3000 as having a yellow tinge, and those corresponding to 1:4000 and 1:6000 as having a slight yellow tinge.

MICROSCOPIC EXAMINATION

Leucocyte Count.—If the fluid is clear or only slightly cloudy, prepare a serologic tube (11 x 75 mm.) for each cerebrospinal fluid to be tested. Using a pipette graduated into 0.005 ml., place in each tube 0.005 ml. of Unna's polychrome methylene blue (p. 38). This dye permits simultaneous counting of red and white cells, since it usually leaves erythrocytes unstained.

Shake the tube of cerebrospinal fluid vigorously to obtain uniform dispersion. Using an ordinary 0.2-ml. pipette (sterile if bacteriologic examination is required; otherwise, cleaned as directed under "Preparation of Glassware," p. 247), draw fluid up to the mark 0.1 and, inserting the tip of the pipette in the dye, expel to the 0.2-ml. mark. Discard the residual fluid in the pipette into a container with 2-per-cent cresol compound (*F76*). Shake the tubes to mix the dye with the fluid, stopper, and store in the refrigerator until ready to perform the count.

Use a Levy counting chamber with Fuchs-Rosenthal ruling, which has a capacity of 3.2 cmm. Dilution with the dye reduces the amount of cerebrospinal fluid actually in the chamber to approximately 3 cmm. Shake the tube containing dye and fluid for thirty seconds, tapping gently to obtain uniform dispersion. Charge the counting chamber and allow two to three minutes for the cells to gravitate to the ruled plate. Count erythrocytes, polymorphonuclear neutrophiles, and mononuclear cells in the 256 squares, and record the number of each. Express as a fraction with the denominator "3" to indicate the average content per cubic millimeter. If the results of the examination suggest that the specimen may contain pathogenic microorganisms, flood the chamber with 2-per-cent cresol compound and clean with liquid soap and tap water.

If the fluid is cloudy, shake the specimen vigorously and pour all except approximately 1 ml. into a centrifuge tube. Draw glacial acetic acid up to the 11-mark of a pipette for white cells and expel it. Then fill the pipette with the fluid in the original tube. Shake well and transfer a drop to the Levy counting chamber. Count all the cells in the nine large squares of the ruled areas and multiply by 10/9 to obtain the number of cells per cubic millimeter. Calculate the average from a count of two drops. If the specimen contains so many cells that an accurate count cannot be made by this procedure, dilute the fluid as for a leucocyte count on blood.

Film Preparation.—The method of preparing films from the sediment of specimens of cerebrospinal fluid depends on the type of examination required. If the fluid is clear or nearly so, centrifuge it in a tube with a conical base capped to prevent evaporation, at about 1800 r.p.m. for twenty minutes. Decant the supernatant to a clean tube. Drain the deposited sediment as completely as possible by placing the tube upside down for from three to five minutes in a rack upon blotting paper that has been moistened with 50-per-cent alcohol. Turn the tube upright and allow to stand for about ten minutes. Prepare for each cerebrospinal fluid a slide with a closed ring 0.5–1 cm. in diameter, drawn with a wax pencil. With a thin capillary pipette fitted with a nipple, stir the sediment thoroughly, rinse the walls around the tip with the residual fluid, and transfer the whole sediment to the prepared slide, within the wax ring. Allow to dry; this may be speeded by placing in an incubator at a temperature not higher than 56°C. or near a radiator grill. Too rapid drying is not desirable, however. When completely dry, fix by flooding with absolute methyl alcohol and allow to stand exactly five minutes. Drain and allow to dry spontaneously. Stain with aqueous alcoholic methylene blue (p. 38). Rinse gently with distilled water to avoid dislodging the film.

Examine with oil immersion. Search for contaminating bacteria; they are frequently near the edge of the film. Observe also the relative abundance of red and white cells. This observation may serve as a check on the cell count previously performed. If there is any doubt concerning the identification of cells in the chamber count, such as sometimes occurs when erythrocytes are stained blue, remove the immersion oil from the film with xylol, flood with methyl or acid alcohol until decolorized, rinse with distilled water, drain, and allow to dry. Restain by Giemsa's method (p. 34) and re-examine.

If bacteria are observed and the findings in other tests suggest the possibility of bacterial infection, clean and decolorize the film as previously described, restain by the Gram or Ziehl-Neelsen method (p. 43) and record the type of microorganism.

If the fluid is cloudy and an examination for the incitant of meningitis is required, make a film preparation from the coagulum, if present, and stain by the Ziehl-Neelsen method or a satisfactory modification (p. 43). Centrifuge the specimen for one-half hour at approximately 2000 r.p.m. and make films from the sediment. Obtain the material with a capillary pipette when an adequate amount is not available to permit the use of a loop. When a large quantity is present, be sure that the film is spread over a sufficient area on the slide so that the cells will be evenly distributed and flattened. Stain one film with aqueous alcoholic methylene blue (p. 38) or by the Wright (p. 42) or Giemsa (p. 34) method to determine the types of leucocytes.

When microorganisms or polymorphonuclear leucocytes are found, stain other films by Gram's method (p. 35). When lymphocytes predominate or the history indicates the possibility of tuberculosis, examine for acid-fast bacilli. (See "Examination for Mycobacteria," p. 295.)

If biscuit-shaped Gram-negative intracellular micrococci are present in the films stained by Gram's method, they may be tentatively identified as *Neisseria meningitidis* although occasionally they may be *Neisseria gonorrhoeae*. Extracellular micrococci having the morphology of *Neisseria* are usually meningococci. (See "Examination for Neisseria," p. 284.) If large numbers of polymorphonuclear leucocytes but no bacteria are present, especially in specimens received from a distance, the probability of a meningitis incited by meningococci should be considered since these cocci autolize readily. In such cases, examine a number of preparations.

In meningitis incited by streptococci, pneumococci, or influenza bacilli, these microorganisms are usually present in large numbers in spinal fluid unless the specimen was collected soon after the onset of symptoms.

Confirm all microscopic findings by cultural or serologic tests. Employ the microserologic test (Neufeld) to determine the type of microorganisms having the morphology of pneumococci. (See "Examination for Pneumococci," p. 273.)

When microorganisms having the morphology of meningococci, streptococci, or pneumococci are found, or when a type of pneumococcus is demonstrated, the physician in charge of the patient should be notified as promptly as possible.

CULTURAL EXAMINATION

If cultural tests are indicated by the results obtained on microscopic examination, follow the methods outlined in chapters dealing with the individual species concerned.

HYDROGEN-ION DETERMINATION

Cerebrospinal fluids submitted through the mail are more alkaline than freshly collected fluids and have a hydrogen-ion concentration usually from pH 8.2 to 8.6. Contamination with acetic acid, or other chemical may, however, alter the pH and render the cerebrospinal fluid unsatisfactory for examination.

To test the hydrogen-ion concentration, use indicators that give optimum differentiation in the range of pH 7.8 to 9.0 (1). Universal indicator, commercially prepared, may also be employed. Place one drop of indicator in a depression in a spot plate. Add one drop of cerebrospinal fluid. Control by tests of solutions of known hydrogen-ion concentration. If the color change is not characteristic, describe the results obtained. Otherwise, record the pH of the fluid.

SEROLOGIC EXAMINATION

When spinal fluid is examined for evidence of syphilis, blood from the patient should be tested at the same time and the findings correlated. (See serologic tests for evidence of syphilis, p. 361.)

Two milliliters of supernatant should be available. It is not satisfactory for the tests if it is purulent or contains fibrin or more than a trace of blood.

COLLOIDAL GOLD TEST (2–7)

Care and Cleaning of Glassware

Glassware used in the colloidal gold test needs special care and treatment. The introduction of traces of copper into the test should be avoided. Do not place pipettes across copper racks or discard them into copper cans. Keep clean pipettes in covered wooden or cardboard boxes. Discard pipettes and specimen tubes in 2-per-cent cresol compound, since there is the possibility of encountering cerebrospinal fluids that contain pathogenic microorganisms. Keep the graduate for redistilled water, the volumetric flask for citrate solution, and the flask for boiling gold sol free from dust and do not interchange with glassware used for other purposes. Rinsing with redistilled water and an occasional steaming should suffice to keep them clean.

Immediately after use, rinse all other flasks, bottles, and tubes in tap water. In addition, completely submerge the tubes—do not allow them to float bottom up. Glassware that comes in prolonged contact with gold sol and that used in the photoelectric colorimeter, however, requires special treatment. Remove gold sol with aqua regia (p. 524) from pipettes and test tubes as soon as possible after use.

Place pipettes with a visible gold precipitate, tip down, for three to five minutes in the storage bottle of aqua regia, keeping the control ends out of the acid. Rinse ten times in tap water and then treat as directed under "Preparation of Glassware," p. 247.

Into each test tube, pipette 3–5 ml. of aqua regia; allow to stand from one to two minutes; then carefully pour back into the storage bottle. Rinse ten times in running tap water, submerge in clear water, and clean as described under "Preparation of Glassware." Keep separately tubes used for the preparation of color standard and all glassware for protected gold sols.

Fill bottles, flasks, and graduates as well as macro tubes for photoelectric colorimeter with aqua regia to slightly above the level at which a precipitate is evident. Allow to stand from one to two minutes. Pour the aqua regia back into the storage bottles. Rinse each piece of glassware ten times with tap water, then twice with distilled water. Steam for ten minutes; invert or lay on paper toweling to cool and dry.

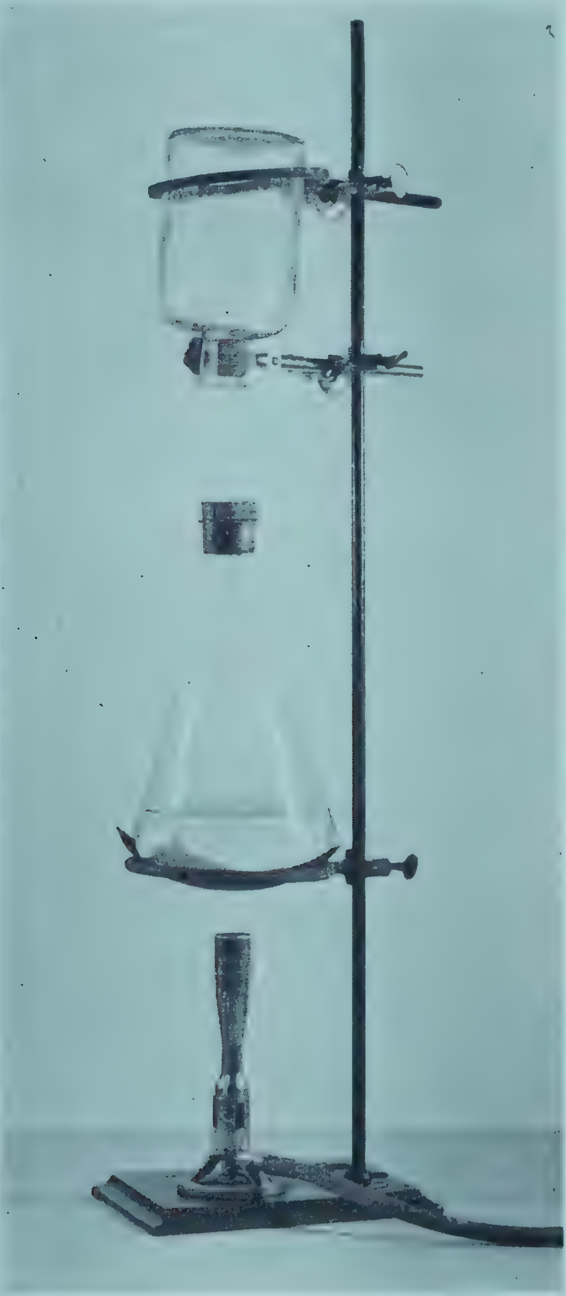

FIG. 54. APPARATUS USED IN THE STEAMING OF GLASSWARE

Method of Steaming.—Use a heavy ring stand and a Fisher burner. Support the 2-liter flask in which the steam is generated over the burner with a 6-inch extension ring and wire gauze with asbestos filter. Fit the flask with a cork stopper through which a hole has been bored and a 6-inch length of 8-mm. glass tubing inserted (see Fig. 54). Boil about 1.5 liters of tap water, and when steam begins to issue from the vent, place the glassware over it. Fasten in place with clamps, springs, or extension rings as required. Steam for ten minutes. As the steam condenses, it should run off freely. A tendency to gather in drops indicates need for further treatment with either a cleaning solution or a detergent. For glassware used for preparation and storage of gold sol, a second treatment with aqua regia is the method of choice. Glassware for special study (e.g., preparation and standardization of buffers) should be steamed before use to remove any possible traces of detergent that may be present.

Reagents

Aqua regia (*8*)

Hydrochloric acid, C.P. (sp. gr. 1.19)	400 ml.
Nitric acid, C.P. (sp. gr. 1.42)	90 ml.
Water, distilled	490 ml.

Prepare in a liter graduate placed in an acid-proof sink. Since heat is generated in the preparation, allow hot water to run into the sink for a short time before placing the graduate in it, to avoid possible breakage due to thermal strain in the glass. See also precautions mentioned under *F78B*. After mixing the solution, pour into a stock bottle fitted with a glass stopper and label. Discard after 6 weeks.

Water, redistilled

Water, distilled	8 liters
Sodium hydroxide, 40-per-cent solution	10 ml.
Potassium permanganate, 1-per-cent solution	10 ml.

Combine and allow to stand at least one week to precipitate any organic matter or metallic salts that may be present.

Procedure.—Prepare redistilled water in a 5-liter Pyrex still with ground-glass connections (Scientific Glass Apparatus Company, No. J-1333 Yoe distilling apparatus, interjoint, pyrex glass) (see Fig. 55).

To prevent the ground-glass stopper from being forced out and broken during distillation, attach it with a cord to the neck of the still. To concentrate and direct the heat around the still, use a Fisher burner and a heating shield. The latter is a cone-shaped piece of sheet metal with a diameter sufficient to allow the still to rest on asbestos strips riveted vertically along the inside of the cone. Attached to the narrow neck is either a metal baffle plate or a wire gauze with asbestos filler, under which is placed the flame. (See Fig. 55.)

Decant 4 liters of the treated water into the reservoir. Add a handful of small pieces of pumice to prevent bumping during boiling. Leave the stopper out of the reservoir until the water has boiled vigorously for ten minutes; then replace, making sure to place a piece of aluminum foil between the stopper and neck to prevent freezing to the still. Allow steam to pass through the condenser coils until the bottom of a 2-liter Erlenmeyer flask is covered with water, then turn on the cooling system. Collect 500 ml. of redistilled water in the flask. (This "first run" should carry off any volatile impurities that may be in the water, and may be used for rinsing the still when distillation is completed.) Then replace with a 3-liter Erlenmeyer flask used only to contain the redistilled water and continue distilling until 3 liters are collected.

Use the redistilled water within 48 hours of preparation.

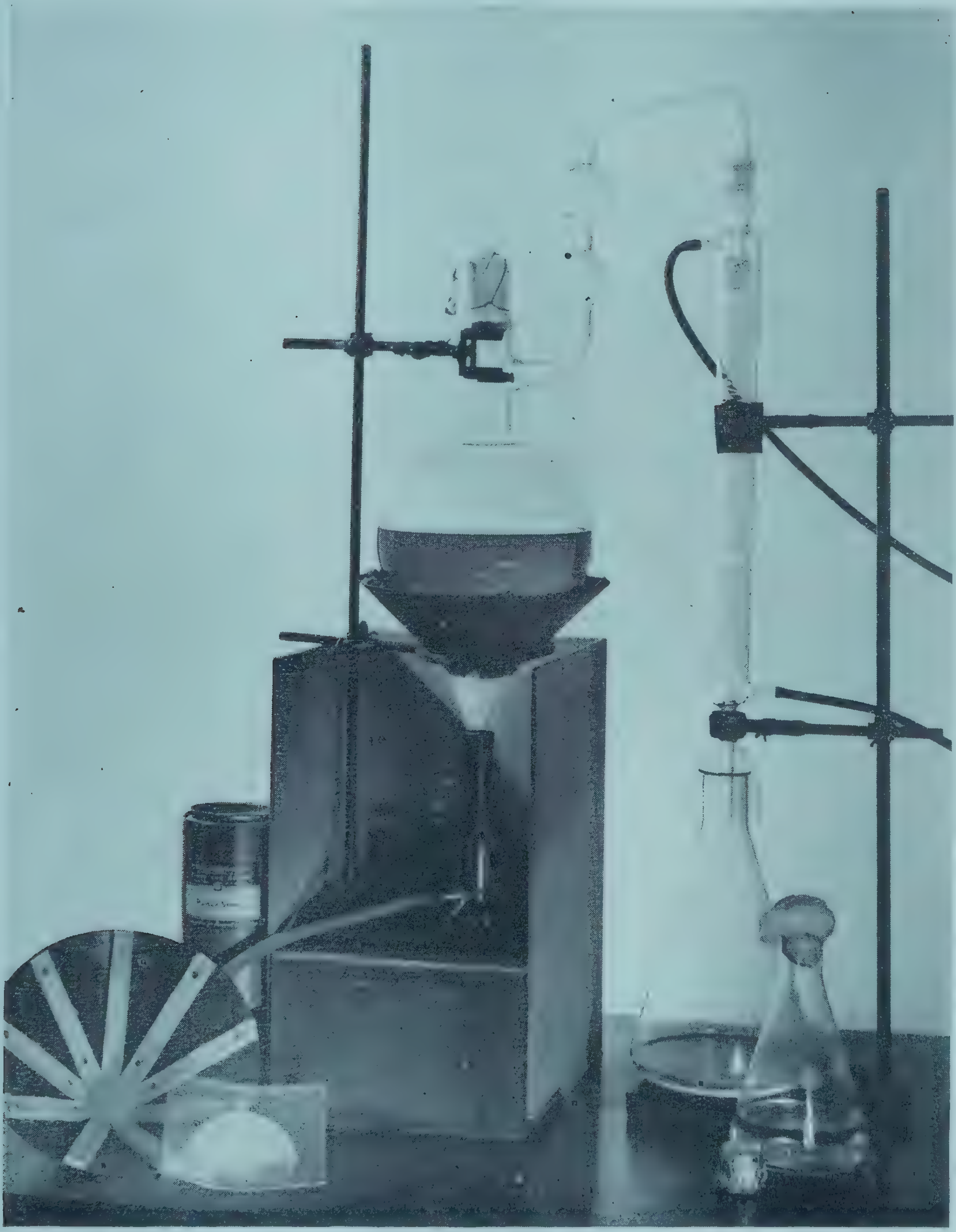

FIG. 55. PYREX STILL WITH FISHER BURNER AND HEATING SHIELD, FOR DISTILLING WATER

Phosphate buffer, pH 7.4 M/21.5

The pH and molar concentration of the buffer in the colloidal gold test is of special importance. A buffer of pH 7.4 $M/21.5$ may be prepared by either the method of Clark-Lubs (9) or that of Sörensen (10).

Clark-Lubs' method

Monopotassium phosphate, 0.2 *M*	100 ml.
Sodium hydroxide, 0.2 *M* (carbonate-free)	79 ml.
Water, distilled	250 ml.

Sörensen's method

Disodium phosphate, anhydrous, Na_2HPO_4, (LaMotte)	5.23 grams
Monopotassium phosphate, KH_2PO_4, (LaMotte)	1.33 grams
Water, redistilled (p. 524), to make	1 liter

Procedure.—The pH should be 7.35–7.45. This may be checked colorimetrically, preferably by use of the Klett-Summerson photoelectric colorimeter with a No. 42 filter, against a standard of pH 7.4 potentiometrically determined. Pipette 6 ml. of known and unknown buffer solutions into separate colorimeter tubes; add 1 ml. *m*-nitrophenol indicator solution (0.30 grams per 100 ml. distilled water) to each and shake. With a tube containing distilled water in place, set the zero point of the colorimeter. Use the same tube to determine the color value of the buffer solutions. The color intensity is increased the greater the pH in the region of 7.4. Some experience with standards of slightly higher or lower pH than 7.4 should indicate the admissible range of variation or standard at pH of 7.35 and 7.45 may be used to give its limits. Adjust pH if necessary, by preparing *M*/21.5 solutions of either the basic (disodium phosphate) or acidic (monopotassium phosphate) salts as required and adding a suitable amount. Check pH as before.

Methocel, 1-per-cent solution

Methocel (Dow Methocel cellulose viscosity 1500 CPS)	10 grams
Water, distilled	1 liter
Formalin	5 ml.

Procedure.—Tear the Methocel into small pieces and place in a 2-liter beaker. Heat approximately 200 ml. of the distilled water in a flask to 80°–100°C. and add to the Methocel a little at a time, stirring constantly with a glass rod. Stir occasionally while cooling. Without removing the rod, place the wet Methocel and the rest of the liter of distilled water in a refrigerator overnight. The next day, pour the chilled water on the cold Methocel gel and mix by stirring and pouring back and forth. Add 5 ml. formalin. Store at refrigerator temperature.

This stock solution is used in the preparation of Methocel I and II in the color standard.

Methocel I

Methocel, 1-per-cent solution	50 ml.
Sodium hydroxide, *N*	8 ml.
Water, distilled	42 ml.

This solution is employed for stopping the progress of coagulation of the blue gold used in the color standard.

Methocel II

Methocel, 1-per-cent solution	50 ml.
Water, distilled	50 ml.

This solution is used for protecting the red gold sol employed in the color standard and in addition furnishes the white of the color standard.

Methocel prevents or stops coagulation of gold sol. Citrate gold sol 1:10,000 contaminated by the faintest traces of a protector would be entirely unsatisfactory for the gold reaction.

Protected red gold sol

Methocel II	2 ml.
Citrate gold sol 1:10,000 (p. 527)	60 ml.

This is used in the preparation of the gold color standard.

Protected blue gold sol

Serum dilution, preserved	0.1 ml.
Phosphate buffer, pH 5.8	3 ml.
Citrate gold sol 1:10,000 (p. 527)	80 ml.
Methocel I (p. 526)	3 ml.

Preserved serum dilution

Pool twenty or more clear, colorless, inactivated sera, from presumably healthy individuals, that do not react in the complement-fixation test for syphilis. Dilute the pool 1:10 with phosphate buffer pH 7.4, $M/21.5$ (p. 525), then add an equal amount of glycerol, so that the final dilution is equivalent to 1:20. This preparation remains sufficiently stable for one month. Different serum pools should yield practically the same results.

Phosphate buffer, pH 5.8

Monopotassium phosphate, 0.2 M	50 ml.
Sodium hydroxide, 0.2 M (carbonate free)	3.7 ml.

Procedure.—Place 3 ml. of Methocel I in a 300-ml. Erlenmeyer flask and set aside. In a second flask, place the preserved serum dilution and the acid phosphate buffer. Mix thoroughly by swinging the flask with a rotary motion. Add the gold sol and allow to coagulate for precisely sixty seconds. Then pour the coagulated mixture upon the Methocel I and mix thoroughly to halt further progress of the coagulation.

This solution is used in the preparation of the gold color standard.

Citrate gold sol 1:10,000

Gold chloride, 1.5-per-cent solution

Gold chloride (Baker's purified, 15-grain ampules)	90 grains
Water, freshly redistilled (p. 524)	400 ml.

Store in amber bottle with ground-glass stopper in the dark.

Sodium citrate, 30-per-cent solution

Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$ crystals, C.P.	30 grams
Water, freshly redistilled (p. 524) to make	100 ml.

Place exactly 30 grams of sodium citrate crystals in a certified (Bureau of Standards) Pyrex 100-ml. volumetric flask. Add freshly redistilled water to make 100 ml. Store in a Pyrex Erlenmeyer flask with a suitable stopper.

Sodium citrate, 1.5-per-cent solution

Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$, 30-per-cent solution	5 ml.
Water, freshly redistilled (p. 524), to make	100 ml.

With a clean precisely calibrated pipette, measure 5 ml. of the 30-per-cent sodium citrate solution into a certified Pyrex 100-ml. volumetric flask and dilute to 100 ml. with freshly redistilled water. Prepare this 1.5-per-cent solution on the day it is to be used.

Procedure.—Use a specially reserved 2-liter Florence flask with a cork-sheathed neck to facilitate handling. (Beakers are inadequate since evaporation during boiling must be kept as uniform as possible; the use of a reflux condenser, however, is unnecessary.)

To shorten the time required to bring the liquid to a boil, use a heating shield and Fisher burner (see Fig. 55). As a special precaution, just before preparing gold sol, boil 1–1.5 liters of redistilled water in the flask for ten minutes and discard. Refill with 1.5 liters of redistilled water and bring to a rolling boil. Add 10 ml. of the 1.5-per-cent gold chloride solution with a clean, precisely calibrated, Pyrex volumetric pipette. Remove the flask from the flame, mix thoroughly with a swing-rotary motion, and replace. Add 10 ml. of the 1.5-per-cent sodium citrate solution and mix as previously described. Reduce the flame and boil gently. First a blue color appears, which, after some time, sharply changes to red. Continue boiling for exactly two minutes after the red color has appeared.

Rinse a 2-liter Pyrex bottle with a small portion of the hot gold sol, which is then discarded. Pour the rest into the bottle and stopper, while hot, with a cotton plug covered with cheesecloth. Cool for one-half hour in a vat of cold tap water; do not place under running tap water where various parts will cool irregularly. When cool, replace the plug with a ground-glass stopper.

If more than one batch of citrate gold sol is prepared on the same day, use the same flask and pipettes throughout but rinse the hot flask with warm redistilled water each time before starting the process. Pool satisfactory batches of citrate gold sol, and determine the transparency.

Gold sols of fairly uniform sensitivity are easy to obtain. Noticeable fluctuations in the freshly prepared sols may, however, occur; these are almost invariably due to the introduction of impurities in the distilled water. A less sensitive gold sol may be recognized with the unaided eye, by its abnormal transparency. A more accurate method is to determine its transparency with the Klett-Summerson photoelectric colorimeter with the red filter No. 66. Use macro tubes, standardize as for micro tubes (see p. 534), and set the zero point of the colorimeter with distilled water. Satisfactory citrate gold 1:10,000 gives initial readings between 120 and 140. If the value is as low as 100, slight transparency may be recognized with the naked eye.

Store in a refrigerator at 9°–15°C. Never introduce a pipette into the storage bottle. Pour into a clean flask, the amount required for pipetting. Discard any that remains in the flask after use. From time to time, determine the turbidity of the stored gold sol.

This reagent may be sent through the mail in Pyrex bottles fitted with ground-glass stoppers coated with paraffin, without perceptible alteration of its reactivity. Before use, treat each bottle with aqua regia and by steaming, as previously described. Fill to the brim to prevent shaking of the colloid.

PROCEDURES

Gold sol used in the test is prepared by reducing gold chloride with sodium citrate. High concentrations of sodium citrate yield relatively hard, insensitive gold sols; low concentrations, a softer reagent. Since acid citrate gold sols age comparatively rapidly, their reactivity must be carefully controlled. A gold sol of medium dispersity (citrate gold sol 1:10,000) is used. The pH and molar concentration of the milieu in the test itself are kept constant and in optimum adjustment to the selected dispersity. For the preparation of citrate gold sol 1:10,000 (p. 527), water redistilled in glass is used (p. 524). Gold sol obtained by the citrate method with distilled water from a metal still is usually red but due to traces of copper may have a low turbidity.

TABLE 63

Color standard

SERIAL NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*
White	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Red	4.75	4.50	4.25	4.00	3.75	3.50	3.25	3.00	2.75	2.50	2.25	2.00	1.75	1.50	1.25
Blue	0.25	0.50	0.75	1.00	1.25	1.50	1.75	2.00	2.25	2.50	2.75	3.00	3.25	3.50	3.75
Color value	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75

SERIAL NO.	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*
White	1.0	1.0	1.0	1.0	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
Red	1.00	0.75	0.50	0.25	0	0	0	0	0	0	0	0	0	0	0
Blue	4.00	4.25	4.50	4.75	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0
Color value	80	85	90	95	100	110	120	130	140	150	160	170	180	190	200

Preparation of Gold Color Standard.—A fresh color standard must be prepared every working day, using 140 ml. of citrated gold sol (1:10,000) (p. 527). Select tubes of uniform diameter to facilitate color matching. Prepare the following fluids: white (Methocel II, p. 526), protected red gold sol and protected blue gold sol (p. 527). Pipette in the order mentioned and in amounts indicated in table 63. The color values in the first twenty tubes up to 100 per cent blue, are derived from their percentual content of blue because, in this range, a greater blue content demonstrates a higher degree of coagulation. The blue-white mixtures, tubes 21–30, correspond to a stronger coagulation than represented by the color value 100 and receive, accordingly, similar numerals above 100. The numerical values are relatively arbitrary, but since they are reproducible to a marked degree, they permit quantitative standardization. When the color values are recorded, the numbers are divided by 10, giving a range from 0.5 to 20.

Preparation and Use of Standard Test Fluid.—As an adequate and accurately reproducible test fluid, a serum pool may be used, similar to that for the preparation of the blue gold for the color standard. Pool at least twenty inactivated sera that do not react in serologic tests for evidence of syphilis and exhibit no hemolysis, chylous properties, bile, or noticeable turbidity. With a dry volumetric pipette calibrated "to contain," pipette 5 ml. of the serum into a dry 250-ml. volumetric flask. Bring to 250 ml. with phosphate buffer, pH 7.4. After careful mixing, add a measured amount of this 1:50 dilution of the serum to an equal amount of glycerol. This 1:100 dilution of the serum in phosphate buffer and glycerol is standard for the colloidal gold test. For use, pipette, in duplicate, 0.1 ml. and add 1.4 ml. of the buffer, pH 7.4. In the same manner as for cerebrospinal fluid, dilute, add 2.5 ml. of citrated gold sol 1:10,000, and read with the gold color standard after two hours. If all the conditions of the test are satisfactory—clean glassware, gold sol of adequate sensitivity, buffer of correct pH and ionic strength—the reaction with the test fluid will yield a constant standard curve.

Perform the control on reproducibility with the test fluid each day colloidal gold tests are made and start the examination of the cerebrospinal fluids only after a standard curve has been obtained in the control.

Technic and Reading of the Test.—Prepare ten serial dilutions of each cerebrospinal fluid, beginning with 1:15, the common ratio of the geometric series being 2/3. Use ten clean Pyrex tubes (100 by 14.5 mm.) for each cerebrospinal fluid. In the first tube, dilute 0.1 ml. of fluid with 1.4 ml. phosphate buffer of pH 7.4 and *M*/21.5 concentration. (If the cerebrospinal fluid has been preserved in an equal volume of 50-per-cent glycerol, use 0.2 ml. of the glycerolized spinal fluid plus 1.3 ml. of buffer.) Place 0.5 ml. of the buffer, pH 7.4, in tubes 2–10. With a 1-ml. Ostwald-Folin Pyrex pipette calibrated "to contain," mix the material in the first tube thoroughly by drawing it up and expelling it at least three times. Transfer 1 ml. to the second tube and repeat. When the material in the last tube is mixed, discard 1 ml. and the pipette. The ten tubes will then contain 0.5 ml. each. In this way, the geometric series of dilutions is obtained: 1:15, 1:22.5, 1:33.7, 1:50.6, 1:75.9, 1:113.8, 1:170.7, 1:256, 1:384, 1:576, which are usually sufficient. In the routine procedure, the dilution is recorded as the next whole number. In fluids from patients with meningitis, the maximum reaction may not be obtained in the tenth dilution. Also, the extent of the reaction with specimens from patients with paresis may require the use of higher dilutions.

To each 0.5 ml. of these serial dilutions, add 2.5 ml. of the citrated gold sol, 1:10,000. Mix by shaking the tubes individually as soon as the gold sol has been added to all ten tubes of one test. Read the test after two hours at room temperature by comparing each tube with the color standard. Divide the color value readings by 10, giving recorded values from 0.5 to 20.

For matching the colors encountered in the test with those of the color standard, white or blue light of not too marked intensity is required; on clear days, a white blind drawn before the window is convenient. Blue daylight bulbs in a lamp that screens off the direct light provide an adequately colored light; the intensity may be regulated by several sheets of tissue paper. The matching of the colors is easy, as shown by duplicate determinations.

TABLE 64

Examples of curves designated as A obtained with normal cerebrospinal fluids

	DILUTION										TOTAL COLOR VALUE	SLOPE OF CURVE
	1:15	1:23	1:34	1:51	1:76	1:114	1:171	1:256	1:384	1:576		
Color value......	2.5	3.5	4.5	5	4.5	4	3.5	2.5	2	1.5	33.5	2.0
Color value......	1	2	3	4	2.5	2.5	2.5	1	1	0.5	20	4.0

Evaluation of the Test

Record the degree of coagulation in each tube of the colloidal gold test. Add these values together to obtain the sum total. The dilution in which the greatest degree of coagulation occurs is designated as the "maximum." Divide the numeral representing the maximum by that designating the reaction in the first tube, to obtain the "slope ratio." These factors, sum total, location of maximum coagulation, slope ratio, and sometimes the degree of coagulation in the first tube, are factors that determine the classification of the types of curves.

With specimens of normal cerebrospinal fluid, the maximum reaction is obtained in the fourth (1:51) or fifth (1:76) dilution of the fluid. The sum total of the reaction is usually less than 45. The slope ratio is of little significance, but is generally less than 4; curve A. (See table 64.)

When inflammatory or other lesions occur in the central nervous system that affect the cerebrospinal fluid, alteration of the normal colloidal gold reaction can be expected. The results of parenchymatous degeneration, proliferation and exudation as well as the presence of blood plasma resulting from hemorrhage or transudation (block) may each affect the findings obtained.

When the inflammatory reaction is productive in type and the vascular permeability is not markedly altered as in chronic neurosyphilis, the maximum coagulation is obtained in the fourth (1:51), fifth (1:76), or occasionally the sixth (1:114) dilution of the fluid. The slope ratio is between 1.4 and 2.1 (or rarely slightly higher). The sum total of the reactions in the ten dilutions of the fluid is usually between 60 and 90; curve B. (See table 65.)

TABLE 65

Examples of curves designated as B
(Formerly classified as second zone or syphilitic curve)

	DILUTION										TOTAL COLOR VALUE	SLOPE OF CURVE
	1:15	1:23	1:34	1:51	1:76	1:114	1:171	1:256	1:384	1:576		
Color value....	6	8	8.5	9	7	7	5.5	5	3	1.5	60.5	1.5
Color value....	5	7	8	9	10	9.5	9	9	7.5	4	78	2.0
Color value....	5	7.5	7.5	8	7.5	6	5.5	4.5	3.5	2	57	1.6

TABLE 66

Examples of curves designated as C
(Formerly classified as third zone, hematogenous, or meningitic curve)

	DILUTION										TOTAL COLOR VALUE	SLOPE OF CURVE
	1:15	1:23	1:34	1:51	1:76	1:114	1:171	1:256	1:384	1:576		
Color value..	2	2.5	4	4.5	6	6.5	5	4	3.5	2	40	3.2
Color value..	1.5	2.5	3.5	5	7.5	10	12	8.5	8	5.5	64	8.0
Color value..	2	3	3.5	5	5.5	7	11	13	9	8.5	67.5	6.5
Color value..	0.5	1	2	3.5	4	5	6.5	9	10	9.5	51	20

When blood colloids gain access to the cerebrospinal fluid, as occurs when there has been exudative inflammation or noninflammatory transudation, the maximum reaction occurs in the sixth (1:114) or a higher dilution. The slope ratio may vary from 2.1 to 20. Such reactions may be obtained with cerebrospinal fluids from patients with purulent types of meningitis, tuberculous meningitis, hemorrhage, and block. (See table 66, curve C.)

When the reaction is maximum in most or all of the dilutions of the fluid, especially in the lower dilutions that are recorded to the left of the series, a plateau type of curve designated as D is obtained. The sum total of the reactions with the ten dilutions tested, varies from 90 to 180 or more. The slope ratio is usually between 1.0 and 1.4. This type of reaction occurs in general paresis (6) and is also obtained with specimens from patients who have other clinical types of neurosyphilis, are asymptomatic, or have multiple sclerosis or some types of virus encephalitis. Consequently, such findings are believed to be of major diagnostic importance. (See table 67.)

TABLE 67

Examples of curves designated as D
(Formerly classified as first zone or paretic curve)

	DILUTION										TOTAL COLOR VALUE	SLOPE OF CURVE
	1:15	1:23	1:34	1:51	1:76	1:114	1:171	1:256	1:384	1:576		
Color value......	18	18	18	18	18	18	18	18	16	8.5	168.5	1.0
Color value......	13	15	15	16	9	7.5	6	5	3.5	2	92	1.2
Color value......	18	18	18	18	18	18	18	18	18	16	178	1.0

TABLE 68

Examples of curves designated as A/B
(This is a type of curve indicative of slight abnormality)

	DILUTION										TOTAL COLOR VALUE	SLOPE OF CURVE
	1:15	1:23	1:34	1:51	1:76	1:114	1:171	1:256	1:384	1:576		
Color value.....	3.5	4.5	6	6.5	5.5	5	4	3.5	3	2	43.5	1.9
Color value.....	4	5	6.5	7.5	7.5	8	5.5	5	4	3	56	2.0
Color value.....	3.5	4	4.5	6	7	6	5.5	5	4	2.5	48	2.0

Between the findings obtained with specimens of normal cerebrospinal fluid and those indicative of chronic productive inflammatory changes, are the ones resulting from slight abnormalities where the vascular permeability is unaltered. Usually, the maximum reaction occurs in the fourth (1:51) or fifth (1:76) dilution, rarely in the sixth (1:114), and the sum total of the findings in all ten dilutions is between 40 and 60. The slope ratio is usually between 1.4 and 2.0; curve A/B. (See table 68.)

When a normal or nearly normal cerebrospinal fluid is contaminated with hemoglobin or bacteria, maximum coagulation may occur in the second (1:23) or third (1:34) dilution of the fluid. The sum total may not be materially altered: the significance of such curve is indeterminate. In addition to the types of curves illustrated, various modifications and combinations are encountered.

PROTEIN DETERMINATION

The micro-Kjeldahl method affords the most dependable technic of determining exactly the very small amounts of protein present in cerebrospinal fluid. (See "Other Methods of Quantitative Chemical Analysis and Precision Tests," p. 121.) Although it cannot be recommended as a convenient routine method, it is the criterion against which other methods must be evaluated. When a photoelectric colorimeter is available, the quantitative turbidimetric test is used; otherwise a quantitative Heller ring test may be employed. In both procedures, the calculation factor, permitting the conversion of the findings of the test into milligrams of protein per 100 ml., must be obtained by comparison with the results of micro-Kjeldahl determinations.

Quantitative Turbidimetric Test

With the Klett-Summerson photoelectric colorimeter, use the blue filter #42 and micro tubes. Operate the instrument in accordance with the directions furnished by the manufacturer. The micro tubes must be clean and standardized.

Cleaning of Micro Tubes.—To avoid inaccuracies in the results of the test due to fingerprints or dust on the outside of the tube, half fill a small beaker with 95-per-cent alcohol. Immediately before inserting the tube in the chamber, dip the lower half into the alcohol and wipe dry with clean, soft, lintless tissue. After tubes have been used, rinse each one at least ten times with tap water and twice with distilled water. Invert to drain. Place upright in a drying oven for approximately an hour or in an incubator maintained at 35°–37°C. overnight, until completely dry. Before use inspect for "watermarks"; if present on the outside, clean with 95-per-cent alcohol; if inside, rinse and dry again as described. New tubes and tubes that remain dirty after two rinsings require treatment with cleaning mixture (*F78A*) or *F78B*). Tubes in routine use should be filled weekly with cleaning solution and allowed to stand for at least fifteen minutes. They cannot be boiled or steamed because of their construction.

Standardization of Micro Tubes.—Use an arbitrary solution of cobalt chloride and the red filter #66. Dissolve 50 grams of the salt in approximately 250 ml. of distilled water. In one micro tube, place 2 ml. or more of distilled water. Use this to set the zero. Place 2 ml. or more of cobalt chloride solution in each of the other micro tubes. Test each tube in turn in the colorimeter, recording the value on the reading scale. Recheck the zero and obtain a second reading for each tube. Group the tubes with the same readings. Clean one tube in each set and refill with distilled water. Set the zero by this tube and record the deviation caused by each tube that originally gave the same reading with the cobalt chloride solution. Check also the readings obtained against this zero with tubes giving different original readings, to determine whether any may be similar to the series being tested. Use each tube in turn for setting the zero. Repeat this procedure until well-defined sets of uniform tubes are established, then etch the tubes so that those of the same series may be readily identified (e.g., A1, A2, etc.). When testing cerebrospinal fluid, use tubes from the same series for performing the test and for setting the zero.

Determination of Calculation Factor.—The test requires the mixture of cerebrospinal fluid and sulfosalicylic acid in definite proportions within the range where turbidity reading is not altered by flocculation. From the reading in the photoelectric colorimeter, the protein content may be calculated in milligrams per 100 ml. The optimum method for determining the calculation factor for the instrument is by direct comparison with the micro-Kjeldahl determination in a series of tests. For example, if 0.6 ml. of a cerebrospinal fluid containing 30 mg. of protein per 100 ml. gives a scale reading of 137, the calculation factor is 0.22. When the use of a series of cerebrospinal fluids is not practicable, three to five pools of normal serum, previously tested by the micro-Kjeldahl method and diluted with 15-per-cent sodium chloride solution so that the protein content is 70 ml. per 100 ml., may be tested in varying, but accurately measured, amounts. Divide the protein content by the reading to obtain the calculation factor. Relate the amount pipetted to the calculation factor to determine the dilution factor. For example, if 0.6 ml. gives a reading of 318 and 0.4 gives a reading of 212, the calculation factor for 0.6 ml. (0.22) must be multiplied by 1.5 (the concentration factor) to obtain the value necessary for 0.4 ml. (0.33).

Determination of Protein Content.—Avoid using amounts of cerebrospinal fluid that contain sufficient protein to cause flocculation; the results of the colloidal gold curve indicate the probable range of the protein concentration that will be needed.

1. If the results of the colloidal gold test are in the range of normal or only slightly abnormal, and there is sufficient material, place 0.6 ml. of cerebrospinal fluid in a standardized micro tube. Add 1.4 ml. of 4.0-per-cent sulfosalicylic acid. Shake to mix and allow to stand ten minutes. Set the zero with distilled water in a micro tube of the same series as that in which the test is performed. Record the deviation caused by the mixture of fluid and sulfosalicylic acid. Take three readings, checking the zero for each. Multiply the reading by the calculation factor to compute the protein content in milligrams per 100 ml. For example, a reading of 113 multiplied by 0.22 indicates 25 mg. of protein per 100 ml.

2. If the colloidal gold test yields a curve with maximum coagulation in the fourth through the seventh dilution, with a gross sum total 45 to 60, mix 0.4 ml. of cerebrospinal fluid with 1.6 ml. of 4.0-per-cent sulfosalicylic acid. Proceed as previously indicated. Multiply the reading by 1.5 (the

concentration factor) as well as by the calculation factor. For example, a reading 158 multiplied by 1.5 (the concentration factor) and then by 0.22 (the calculation factor) indicates 50 mg. of protein per 100 ml.

3. Fluids that react to a more marked degree (e.g., those giving evidence of the presence of considerable protein of hematogenous origin, or those showing marked coagulation—color value at or approaching 18—in each tube) must be tested in greater dilution. For example, if 0.2 ml. of a fluid giving a gross sum total of 175 in the colloidal gold test is mixed with 1.8 ml. of 4-per-cent sulfosalicylic acid and gives a reading of 155, this reading multiplied by 3 (the concentration factor) and 0.22 (the calculation factor) indicates that the fluid contains 102 mg. of protein per 100 ml.

The ratio of the sum total obtained by adding the numerical values expressing the degree of coagulation in each tube of the colloidal gold test and the protein content expressed in milligrams per 100 ml. may furnish information of diagnostic significance. With specimens of normal cerebrospinal fluid, the usual value is found to be about 1.5; with those from nondegenerative neurosyphilis, approximately 2.0. When an exudative inflammatory process is present, the value is usually less than 1.0 and is of more significance as the decimal becomes smaller. When parenchymatous degeneration occurs, the ratio is greater than 1.0. The highest value of all is encountered in general paresis.

Quantitative Heller Ring Test

Constant artificial illumination and dark background increase the visibility of the disk of precipitated protein in the Heller ring test, so that the endpoint is sharply defined. To standardize the test so that the results may be expressed in milligrams per 100 ml., a calculation factor must be determined for the individual worker. Since the reading is subjective, the factor will be influenced by acuity of vision. Comparison with the micro-Kjeldahl test affords a desirable control. It is essential, moreover, that the comparison embrace normal as well as pathologic cerebrospinal fluids. At or near the normal range, the results of the Heller ring test are reproducible, but the greater the total protein concentration, the less the accuracy of the Heller test.

Technic and Reading of the Test.—To counteract the effect of minute scratches on the outside of the test tube, apply a light film of paraffin oil before use. To perform the Heller ring test, prepare a 1:5 dilution of cerebrospinal fluid in 0.9-per-cent sodium chloride solution. Mix thoroughly with a 0.2-ml. pipette. Place approximately 1 ml. of nitric acid (specific gravity 1.42) in a tube, 100 by 5 mm. Overlay the acid with 0.15 ml. of the diluted cerebrospinal fluid. After exactly three minutes, place the tube in the bushing of a dark box (11) (Fig. 56) and inspect.

From the 1:5 dilution, prepare higher dilutions as required. For example, a very faint ring at the interface suggests that the endpoint may be reached in a 1:6 or 1:7 dilution, while a heavy ring indicates that a greater dilution is required.

Record the last dilution in which precipitate is observed and also the first dilution which shows no ring (e.g., 1:15+, 1:12−). Multiply the endpoint dilution by the calculation factor to express the protein content in milligrams per 100 ml. For example, if the calculation factor is 3, the result cited would represent approximately 45 mg. per 100 ml. Because of the subjectivity of the method, check the calculation factor periodically, performing the test with a solution of known protein content.

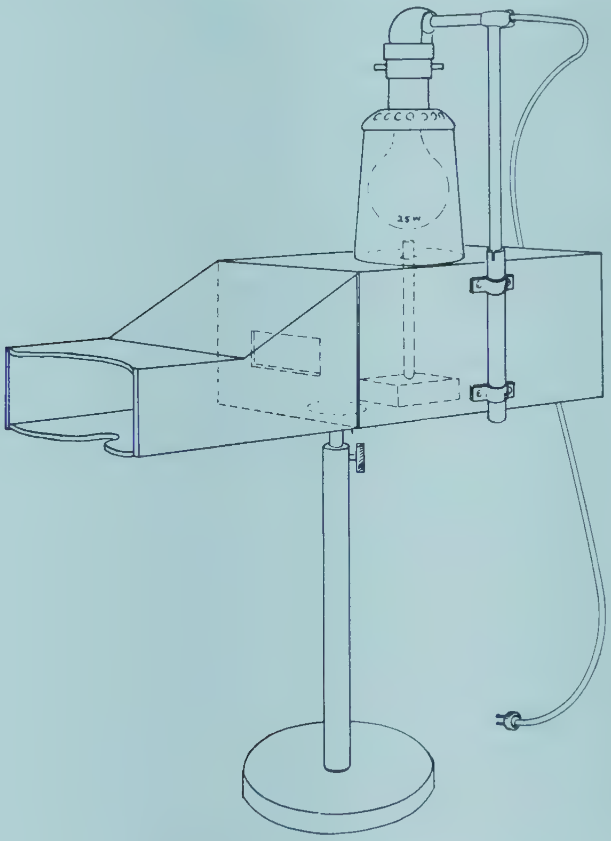

FIG. 56. VIEWING BOX

The viewing box is made of ¼ in. plywood with metal fasteners. The base is cast iron, the tubes are steel, the lamp socket with angle top is standard, and the lamp shade pressed steel.

CORRELATION AND REPORTING OF RESULTS

When all of the individual procedures have been completed and entered on the record form, review the data. Consider first the procedures designed to detect contamination, whether by microorganisms, by admixture of blood, or by alteration of the hydrogen-ion content. Determine to what extent the contamination, if present, rendered the cerebrospinal fluid unsatisfactory for testing.

Review the findings of the cell count, complement-fixation test, colloidal gold test, and protein determination individually and collectively. If the results of any one procedure are at variance with those in the other three, repeat the test that is not in accord.

Report to the physician the results of each test. When the findings warrant, suggest interpretation of their significance.

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CHAPTER 38

HISTOLOGIC PROCEDURES

MUSEUM

PREPARATION OF SPECIMENS

When tissue is received, describe the gross appearance and record the fixative used. Prepare a tag with a string bearing the accession number, date, number of pieces of tissue, and fixative used, so that it can accompany the blocks of tissue until they are sectioned. Cut blocks from characteristic portions of the tissue specimen thin enough so that by rapid methods of fixation, no more than six hours will be required for penetration and fixation.

FIXATION

The most important step in the preparation of tissue sections is the immediate and proper fixation of all tissue as soon as removed in order to preserve the cellular structure and tissue relationships unaltered. A good fixative must therefore act rapidly and penetrate quickly before decomposition has occurred. In the selection of the proper fixative, several points must be considered—the type of tissue to be studied, the speed required in preparing the section, the staining methods to be followed, and the particular object of the examination. Formalin, Zenker, Zenker-formol, and Bouin fixatives are adequate for most work of a routine nature. However, alcohol, alcohol-formol, mercuric chloride-alcohol, and Regaud's fluid are occasionally required.

Unfixed tissue can be examined by the frozen-section method (1) when the findings must be available without delay.

Formalin.—Formalin (p. 547) is the best fixative for routine use and is especially valuable when haste is essential. Small pieces of tissue are usually well fixed in twenty-four hours. Washing is not ordinarily required. Tissue may be left in 10-per-cent formalin for considerable periods of time without deterioration. It may be put in 1-per-cent ammonium hydroxide (26–28 per cent NH_3 by weight) for one to several days to remove the formalin, and can then be refixed in Zenker's solution (p. 550).

Zenker's Solution.—This fixative (p. 550) is especially useful when fine cellular detail is necessary. It is a prerequisite to staining by Mallory's phloxin methylene blue stain (p. 550).

Fix tissues approximately 2 by 3 by 0.5 cm. for twelve to twenty-four hours; wash in running tap water, twelve to twenty-four hours. They are then ready for dehydration. Tissue may be left in 80-per-cent alcohol for preservation indefinitely.

Zenker-Formol Fixative.—This fixative (p. 550) is valuable for a study of tissues of the hematopoietic system as well as of the cellular elements associated with inflammation, and for demonstration of microorganisms. The procedure is the same as for Zenker-fixation.

Bouin Picro-Formol Fixative.—This is an excellent fixative (p. 547) particularly for fresh material. Although preferred for tissue to be stained by Masson's trichrome stains (p. 551), it may be used for other staining methods equally well. Cut blocks of tissue 2 by 3 by 0.5 cm. and place in fixative for one to three days, preferably the latter. Shake occasionally. Transfer to 95-per-cent alcohol.

Alcohol.—Alcohol, absolute or 95-per-cent, shrinks tissue and makes it brittle. However, it is a requisite with stains for glycogen and is useful for demonstrating various microorganisms and certain chemical constituents.

Alcohol-Formol Fixative.—This (p. 546) is satisfactory especially for surgical tissues when haste is necessary. It fixes and partially dehydrates simultaneously.

Mercuric Chloride-Alcohol Fixative.—This fixative (p. 548) is very useful when microorganisms or inclusion bodies are to be stained. Fix tissues for at least forty-eight hours. Then transfer to 95-per-cent alcohol.

Regaud's Fluid.—This is an excellent fixative (p. 549) for human tissue suspected of containing rickettsiae; they are stained sharply and deeply. Fix tissues for forty-eight hours, changing the fluid every twelve hours.

DECALCIFICATION

Fix thin pieces of bone in 10-per-cent neutral formalin (p. 547) for twenty-four hours. Wrap in gauze and suspend in a bottle filled with 5-per-cent aqueous solution of nitric acid. Incubate at not more than 37°C. for twelve to twenty-four hours; change the fluid after twelve hours if decalcification is not complete. Shake occasionally. Prick the tissue with a needle; when it meets no obstruction, the bone is decalcified. If possible, the pricking should be done in a part of the block where the distortion that may result will do the least harm. Wash in running tap water, twelve to twenty-four hours. The tissues are then ready for dehydration.

A method of decalcifying bone *in vacuo*, which completes the process within eight hours has been reported (2). Fix tissues in 10-per-cent neutral formalin, place in a wide-mouthed bottle filled about three-fifths of the volume with 5 per-cent aqueous solution of nitric acid. Change the fluid two or three times during the process. Attach the bottle containing the nitric acid and tissue to the vacuum pump. Protect the pump and rubber tubing from the acid fumes by placing in the filter flask a solution of sodium hydroxide to which a few drops of phenolphthalein has been added. Extend the tubing from the acid to the bottom of the flask and place a safety bottle between the flask and the tissue bottle. Set the vacuum pump in operation. At the end of each hour, stop it, and inspect the tissue. Prick with a needle and as soon as it penetrates all parts of the bone without resistance, stop the process. Neutralize by placing the tissue directly in 2-per-cent ammonium hydroxide solution in distilled water. Attach the vacuum apparatus and operate it for another ten minutes. Place the tissue in 10-per-cent formalin for two hours. Dehydrate and embed in the usual manner.

If Zenker fixation is used instead of formalin, wrap the tissue in gauze and wash from two to four hours in tap water. Place in 80-per-cent alcohol from eight to twenty-four hours, changing the alcohol after two to four hours. Remove from the alcohol, place in 5-per-cent aqueous nitric acid, and proceed as described for formalin-fixed tissue. After neutralizing, however, place the tissue in 80-per-cent alcohol for two hours before dehydrating.

Fix biopsy material from sternal bone marrow in Zenker formol (p. 550). Wash in running tap water overnight and decalcify in Wagoner's solution (p. 549). Wash in tap water, twelve to twenty-four hours, and dehydrate in the usual manner.

DEHYDRATION

Alcohol.—Since water and alcohol are not miscible with paraffin, they must be removed before the tissue can be infiltrated with paraffin. Ascending concentrations of alcohol, followed by xylol, are generally used for dehydrating, hardening, and clearing of tissues. Dioxane, which is miscible with water, alcohol, and paraffin, may be used in place of alcohol and xylol.

Arrange wide-mouthed 3-ounce bottles that can be tightly corked so that the tissue can be passed through the solutions in sequence. A tentative schedule for use with pieces of tissue approximately 2 by 2 by 0.5 cm. is as follows: 80-per-cent alcohol, four hours; 95-per-cent alcohol, six to twelve hours; two changes of absolute alcohol, twelve to twenty-four hours in each; two changes of xylol, six to twelve hours in each.

Dioxane-Paraffin (3–7).—Dioxane (diethylene oxide) is the preferred dehydrating agent since it permits rapid dehydration and clearing of tissues with very little distortion or shrinkage. It is soluble in water, alcohol, xylol, and melted paraffin. Dioxane is toxic and acts as a cumulative poison; in small quantities as used in a laboratory, if proper precautions are observed, it is a negligible hazard.

Any fixative may be used, but for rapid work, 10-per-cent formalin (p. 547) is best. After thorough fixation, and washing of the material if necessary, cut blocks of tissue approximately 5 mm. in thickness to permit rapid penetration. For dehydration, put anhydrous calcium chloride in the bottom of a Stender dish (93 mm. by 62 mm.) to a depth of 1 cm.

Place a perforated porcelain cup in the dish and fill with dioxane to within 1 cm. from the top. Place the tissues in the cup and cover with a tightly fitting ground-glass cover to prevent vapor from escaping. Leave the tissue in the dioxane from six to twelve hours depending upon the thickness. Usually a complete change of dioxane and calcium chloride is made once in every two weeks or sooner if large numbers of tissue blocks are passed through it. Transfer the tissue directly to a bottle containing paraffin and incubate at 54°–55°C. for two to four hours. Change to a second bottle with paraffin and reincubate for the same period. Care should be taken to have the tissue rest a few centimeters above the bottom of the bottle since dioxane, being heavier than paraffin, tends to settle.

EMBEDDING TECHNIC

Tissue must be hardened in order to make thin sections for microscopic examination. The paraffin method, which is generally employed and which is the most useful, permits the preparation of very thin sections. A celloidin technic (8a, 9) is preferred by some histologists. It is especially useful when large sections are desired. The frozen-section method is particularly valuable in the examination of surgical specimens, since sections can be cut promptly after the receipt of fixed or unfixed tissue. It is also used in histochemical examinations for fat, for staining amyloid, in various metallic impregnations of tissue of the central nervous system, and in special cytological technics.

Paraffin Method

Infiltration.—The purpose in using paraffin is to fill natural cavities, infiltrate the tissue, and surround it in a firm block. The clearing fluid must be entirely removed from the blocks of tissue and replaced with paraffin before they are embedded. To do this, transfer the tissues from dioxane to paraffin that is kept in a paraffin bath in an incubator maintained at a temperature of 54°–55°C. and leave for two to four hours. The incubator should be used only for the melted paraffin since traces of moisture in the atmosphere may be detrimental. When dioxane is not used as a dehydrating agent, transfer the tissue from xylol to paraffin and leave approximately four hours.

Embed in a paraffin-wax mixture (p. 549), as suggested by Masson (10a). Pour the paraffin heated to 56°–58°C. into a rectangular paper box of a suitable size. Orientate and completely submerge the tissue in it. Speed is essential. Cool quickly by placing the embedded tissue in a refrigerator.

Cutting Sections.—The preparation of sections that are evenly thin, and not crumpled or ragged, requires a precision microtome either of the rotary or the sliding type, and a heavy expertly sharpened knife. Directions for the use of the microtome are furnished by the manufacturer.

Examine the edge of the knife with a low power of the microscope (× 50) to make sure that it is free from nicks before use.

To sharpen the knife, suspend about 5 grams of Turkish emery No. FFF (A. H. Thomas Co.) in 50 ml. of water and shake. Allow to stand for a moment to permit the coarse particles to settle and then pour over the surface of a clean, heavy glass slab (30 by 45 cm.). Support the knife with a slightly worn guard and move it back and forth for about twenty strokes with uniform pressure. Turn it over and repeat the process. Continue the procedure until the larger nicks are removed; usually fifteen to thirty minutes are required. Wash the knife and glass plate free of emery. Suspend 2–3 grams of white rouge (A. H. Thomas Co.) in 50 ml. of water, and pour over the glass slab. Work the knife with guard back and forth as in the first procedure, for approximately twenty strokes until all the microscopic nicks are removed.

Wash the rouge from the knife and glass plate. Sprinkle a small amount (as much as can be lifted with a knife point) of a diamantine powder, No. 7178 No. 2 medium (A. H. Thomas Co.), on the glass plate and add enough water to keep it moist. Work the knife back and forth, turning it over after each stroke. Continue until the bevel is well polished and a keen edge is obtained.

Trim the paraffin block so that it forms as nearly a rectangular mass as possible. Heat the block holder until it will just melt the paraffin. Fix the cold paraffin blocks to it, immediately immerse in cold water, and cool the face of the block with ice. Clamp a sharp knife in position, just clearing the paraffin block. Tilt the edge slightly towards the block. Adjust the indicator regulating the sections to the required thickness, preferably 5μ.

To cut sections in ribbons, fix the object holder in position by means of the adjusting mechanism so that the broad side of the rectangle is parallel with the edge of the knife. With a camel's hair brush and a hot scalpel, detach a portion of the ribbon and float it, glossy side down, in a rectangular Pyrex dish of water maintained at about 50°C., depending upon the melting point of the paraffin used; in some instances, 40°–45°C. is preferable. The heat flattens the sections. Affix them to glass slides. If the room is too warm or too cold, the sections may be wrinkled or not adhere to each other.

Write the serial number of the specimen on one end of a clean glass slide with a diamond-point pencil. Place a drop of Mayer's egg albumen fixative (p. 548) on the slide and spread evenly over the surface. Remove any excess with the finger. Float the section onto the slide, drain off the water, and place in an upright position in a wooden box without a cover. After four to six hours' incubation at 54°–55°C., the section will firmly adhere to the slide so that it can be carried through various solutions and stained. When haste is necessary, heat the slides carefully, section side up, over a small Bunsen flame until the paraffin begins to melt. By that time, the water will have completely evaporated and the slides can be placed in xylol.

Clearing and Dehydrating.—Before the mounted sections can be stained, the paraffin must be removed. Arrange a series of square dishes containing xylol and descending percentages of alcohol in consecutive order.

Paraffin: Place the slides bearing the sections that are infiltrated with paraffin in racks and pass through two changes of xylol, two minutes in each; two changes of absolute alcohol, one minute in each; then 95-, 90-, 80-, and 60-per-cent alcohol, one minute in each; and finally into distilled water.

Zenker-fixed tissue: After the sections have been through 95-per-cent alcohol, place them in 90-per-cent alcohol containing 0.5 per cent iodine for ten minutes. Wash in tap water. Treat with 5 per cent sodium hyposulfite. Wash in running tap water and rinse in distilled water.

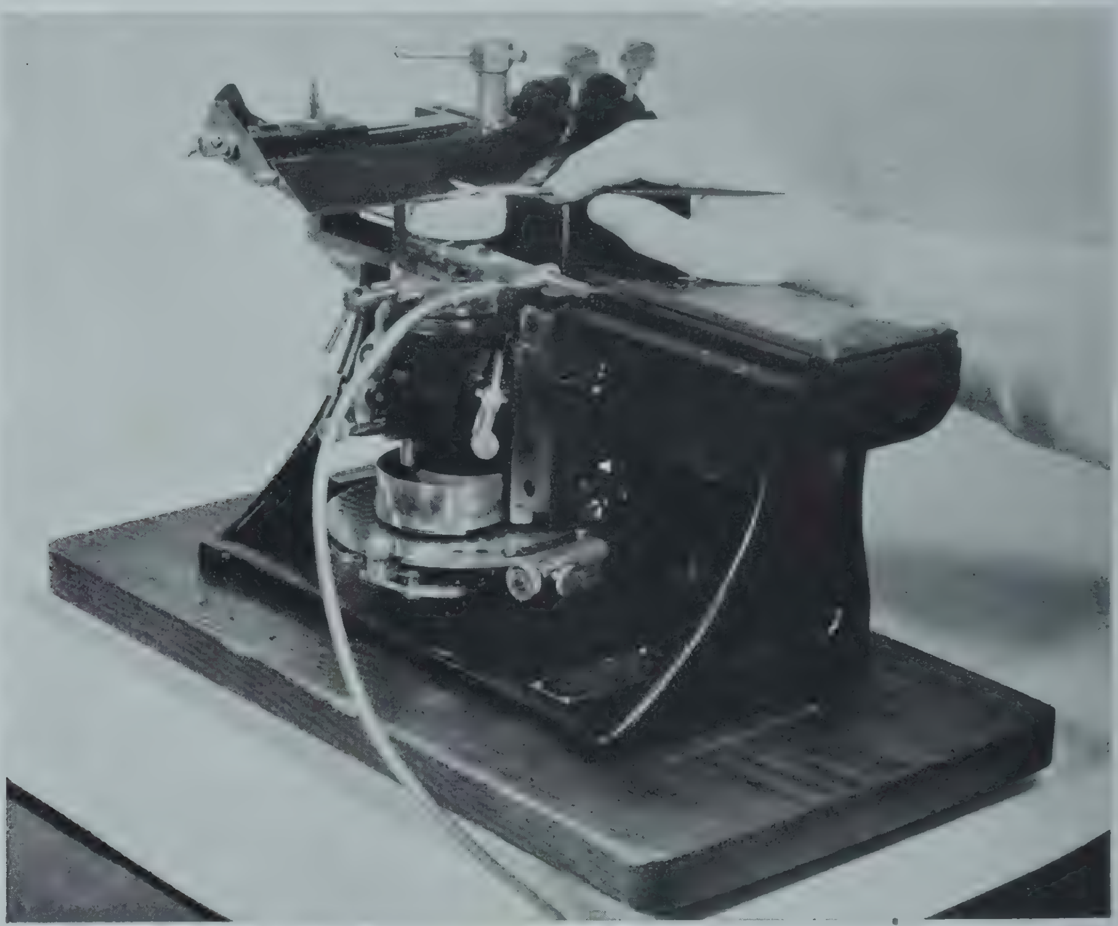

Fig. 57. Microtome with Carbon Dioxide Attachment for Frozen Sections

Frozen-Section Technic (11)

For the best results, use a large sliding microtome and heavy, sharp knife, 240 mm. long. A freezing attachment and carbon dioxide tank are also essential. If needles are required to handle frozen sections, always use those made of glass. See Fig. 57.

Small pieces of tissue should be thoroughly fixed in formalin. Blocks up to 4 by 4 by 1 cm. can easily be frozen, although pieces 2 by 1 by 0.5 cm. are adequate for diagnosis. Place the tissue on the freezing block, making it adhere with a little water. Allow carbon dioxide to escape until the tissue is frozen to a proper consistency. Overfreezing should be avoided. Cut sections 10μ and transfer them with a camel's hair brush to a Stender dish approximately 6 cm. in diameter containing distilled water.

Float sections onto a glass slide. Drop 95-per-cent alcohol on the slide. Blow gently. Repeat the procedure until the sections are dry. Pour a drop of 0.5-per-cent solution of celloidin in ether and alcohol over sections. Drain off excess at once and blow on the sections. Immerse in water to harden. The sections should now be attached to the slides and ready for staining.

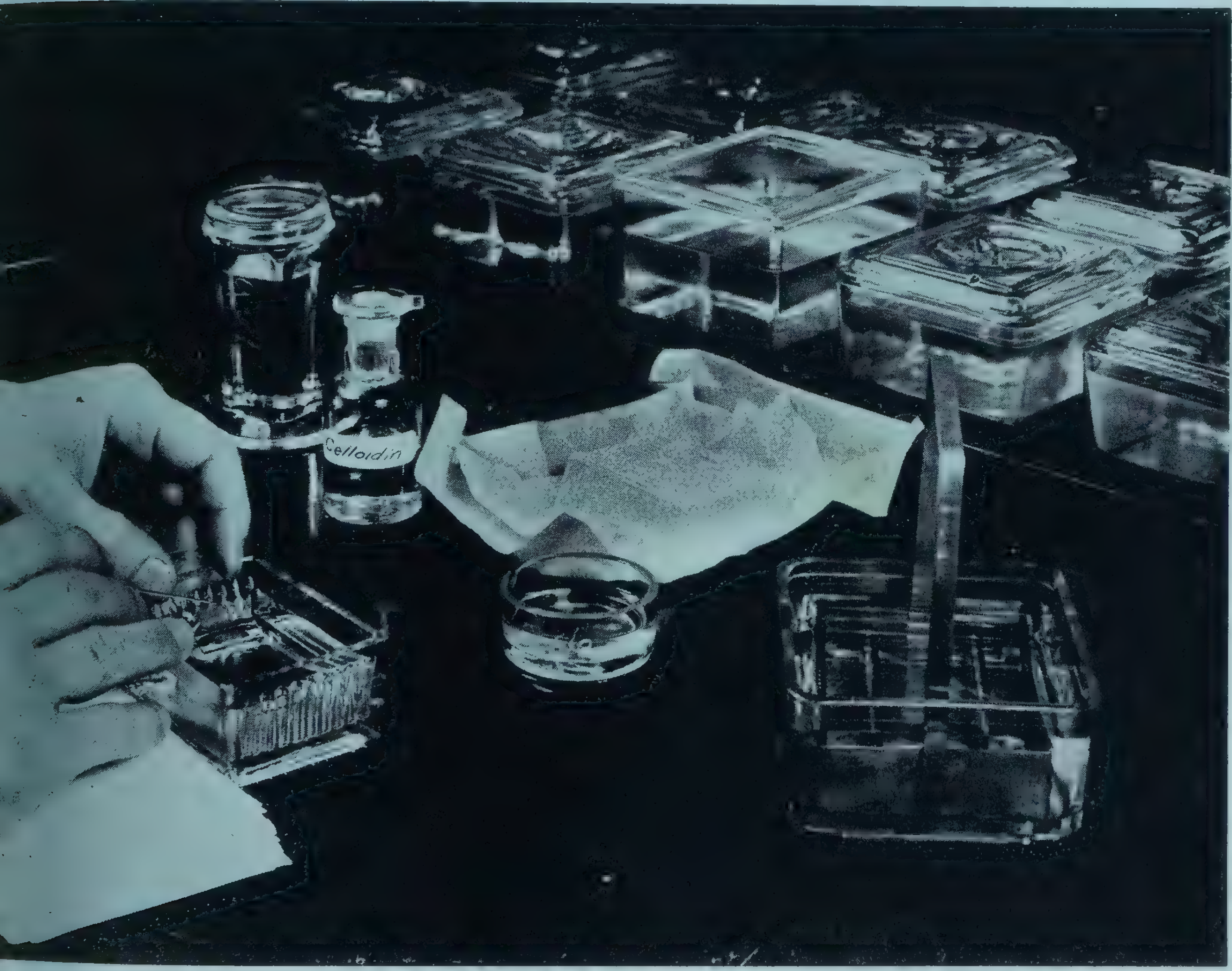

Fig. 58. Equipment and Reagents for Staining Frozen Sections

Stain in Harris's hematoxylin (p. 548) for two to five minutes, depending on the type of tissue. Rinse in distilled water. Wash for five minutes in running tap water, until blue. Place in 80-per-cent alcohol, one minute. Stain in 0.5-per-cent phloxin in 95-per-cent alcohol, one minute. Dehydrate in 95-per-cent alcohol, one minute; two changes of absolute alcohol, one minute in each. Clear in xylol. Mount in neutral xylol-dammar (p. 548).

Rapid Method.—Float sections in a dish of distilled water. With a glass rod, transfer to Harris's hematoxylin (p. 548) and stain for one minute. Rinse in distilled water, thirty seconds; treat with 0.5-per-cent ammonium hydroxide (0.5 ml. concentrated ammonium hydroxide in 100 ml. distilled water) until the sections are blue, ten to fifteen seconds. Wash in tap water, one minute. Pass through 70-per-cent alcohol, thirty seconds; 95-per-cent alcohol, thirty seconds. Stain in 0.5-per-cent phloxin in 95-per-cent alcohol, ten to thirty seconds. Dehydrate in 95-per-cent alcohol, thirty seconds; two changes of absolute alcohol, thirty seconds each. Clear in xylol. Mount in neutral xylol-dammar.

REAGENTS, STAINS, AND METHODS OF STAINING

Stains certified by the Commission on Standardization of Biological Stains (12) and other chemicals of C.P. grade or the equivalent are used unless otherwise specified. Only the highest grade of absolute, neutral, acetone-free methyl alcohol is employed. Formalin is a commercial product, 37- to 40-per-cent formaldehyde (by weight). Hence, 10-per-cent formalin is about a 4-per-cent solution of formaldehyde.

Additional formulas are given in "Stains and Reagents. Methods of Application," p. 30, under "Formulas for Media and Auxiliary Preparations," p. 183, and in other chapters when they are limited to a specific use.

GENERAL REAGENTS AND STAINS

Acid alcohol

Hydrochloric acid (sp. gr. 1.19)	3 ml.
Alcohol, 95-per-cent	97 ml.

Alcohol-formol fixative

Formalin	10 parts
Alcohol, 95-per-cent	90 parts

Asphalt cement—Muir-Judah (for sealing museum jars) (13)

Asphalt, Trinidad Lake (common)	1200 grams
Linseed oil, boiled	170 ml.
Oil of amber	30 ml.

Heat the oils, add slowly 400 grams finely broken asphalt, boil for one-half hour, cool. Add the balance of the asphalt 200 grams at a time; boil for one-half hour, allowing the cement to cool between each addition. Skim the debris. Boil for an additional three-quarters of an hour or until hard enough for use.

Bouin's picro-formol fixative (14, 10b)

Water, distilled	30 parts
Formalin	10 parts
Trichloroacetic acid, 2-per-cent aqueous solution	2 parts
Picric acid, to saturation	

Mix the formalin, water, and trichloroacetic acid solution and add a surplus of picric acid. Shake. Saturation is reached after three days.

Clarite X, a mounting medium

Clarite X	60 grams
Toluene	46.2 ml.

Place the Clarite X in a small bottle and add the toluene. Shake occasionally until dissolved; approximately twelve hours are required.

Delafield's hematoxylin (15)

Hematoxylin	4 grams
Alcohol, 95-per-cent	25 ml.
Aluminum ammonium sulfate, $AlNH_4(SO_4)_2\cdot 12H_2O$, saturated aqueous solution	400 ml.

Dissolve the hematoxylin in the alcohol and add to the aluminum ammonium sulfate in a wide-mouthed bottle. Stopper lightly with cotton and allow to stand in a light place for about two weeks. Filter through paper and add:

Glycerol	100 ml.
Alcohol, 95-per-cent	100 ml.

Allow the mixture to stand in the light until it assumes a deep magenta color. Then filter and keep in a tightly stoppered bottle. Dilute as desired, approximately 1:5, before use.

Ehrlich's aniline crystal violet (16)—modified

Solution I

Crystal violet (85 per cent dye content)	2.5 grams
Alcohol, 95-per-cent	12 ml.

Solution II

Aniline	2 ml.
Water, distilled	98 ml.

Shake, allow to stand, and filter.
Mix solutions I and II.

Formalin, 10-per-cent, fixative

Water, tap	2700 ml.
Formalin, commercial	300 ml.

Place about 5 grams of calcium carbonate in the bottom of the container to neutralize the formalin.

Harris's alum hematoxylin (17)

Hematoxylin	1 gram
Alcohol, absolute	10 ml.
Aluminum ammonium sulfate, $AlNH_4(SO_4)_2 \cdot 12H_2O$	20 grams
Water, distilled	200 ml.
Mercuric oxide	0.5 gram

Dissolve the hematoxylin in the absolute alcohol and the alum in the distilled water. Add the alum mixture to the dissolved hematoxylin and bring to a boil as rapidly as possible. Add the mercuric oxide. The solution becomes a dark purple immediately. Plunge the vessel with contents into cold water; cool rapidly.

This preparation needs no ripening and keeps well. Before use, add to each 75 ml. of the stock solution:

Acetic acid, glacial	5 ml.
Glycerol	25 ml.

Kaiserling solutions—Lundquist-Robertson modification (18, 19)

(for museum specimens)

Solution I

Potassium acetate	850 grams
Potassium nitrate	450 grams
Chloral hydrate	800 grams
Formalin	4445 ml.
Water, tap	40,000 ml.

Solution III

Potassium acetate	4000 grams
Chloral hydrate	2000 grams
Glycerol	4000 ml.
Water, tap	36,000 ml.

Mayer's egg albumen fixative

Egg white	50 ml.
Glycerol	50 ml.
Sodium salicylate	1 gram

Beat and filter through cheesecloth.

Mercuric chloride—alcohol fixative

Mercuric chloride saturated aqueous solution (7 grams per 100 ml. distilled water)	2 parts
Alcohol, absolute	1 part

Neutral xylol-dammar (for mounting sections)

Place approximately 10 ounces of dammar in a tall glass cylinder and cover with neutral xylol. After the dammar has dissolved, pass the mixture through coarse filter paper into a 1-liter graduate. Add 2 grams of calcium carbonate and let settle to the bottom of the graduate. Cover with two layers of gauze and allow to stand at room temperature until the mixture becomes clear. Pour off the clear fluid into a second 1-liter graduate at the bottom of which are deposited approximately 2 grams of calcium carbonate. Cover the top with gauze and place in a paraffin incubator where evaporation takes place slowly. This requires from one to two months. When the clear xylol mixture has the consistency of thin syrup, store it in brown-glass tightly stoppered bottles. This forms an excellent mounting medium.

Paraffin (for embedding) (10a)

Paraffin	90 parts
Beeswax, pure	10 parts

The beeswax gives the mixture a consistency that allows sections to be cut easily.

Regaud's (Müller's) fluid (20, 21, 8e)

Potassium dichromate	25 grams
Sodium sulfate	10 grams
Water, distilled	1000 ml.

Add 20 ml. of formalin to each 100 ml. of fixative before use.

Schaudinn's fluid (22)—modified

Mercuric chloride, saturated solution in 0.85-per-cent salt solution (7 grams per 100 ml.)	65 ml.
Alcohol, 95-per-cent	33 ml.
Acetic acid, glacial	2–5 ml.

Wagoner's solution—adapted from Bock (23–25a) (for decalcification)

Solution I

Formic acid, 85–90-per-cent	50 ml.
Water, distilled	35 ml.

Solution II

Sodium citrate, $Na_3C_6H_5O_7 \cdot 2H_2O$	17 grams
Water, distilled, warm	85 ml.

Mix solution I and add to solution II.

Weigert's iron hematoxylin (26)

Solution I

Hematoxylin	1 gram
Alcohol, 95-per-cent	100 ml.

Allow to ripen several months.

Solution II

Ferric chloride, $FeCl_3 \cdot 6H_2O$, 30-per-cent aqueous solution	4 ml.
Hydrochloric acid (sp. gr. 1.19)	1 ml.
Water, distilled	95 ml.

Mix equal volumes of solutions I and II each time before use. The iron hematoxylin is satisfactory while the color is black or purple; unsatisfactory, when brown.

Zenker's solution (27)

Potassium dichromate	2.5 grams
Mercuric chloride	8 grams
Water, distilled	100 ml.

Dissolve the dichromate and mercuric chloride in water by heating. Cool. (Some of the chloride will precipitate.) Just before use, add 3–5 ml. of glacial acetic acid to each 100 ml. of the stock solution.

Zenker-formol fixative (28)

Potassium dichromate	2.5 grams
Mercuric chloride	8 grams
Water, distilled	100 ml.

Dissolve the dichromate and mercuric chloride in water by heating. Cool. (Some of the chloride will precipitate.) Just before use, add 10 ml. of formalin to each 100 ml. of the stock solution.

MICROHISTOLOGIC METHODS

Hematoxylin-eosin method

Eosin

Eosin Y, water soluble	0.5 gram
Alcohol, 95-per-cent	25 ml.
Water, distilled	75 ml.

Add a crystal of thymol. Filter before use.

Procedure.—Follow the method for hematoxylin phloxin, substituting eosin for the phloxin.

Hematoxylin-phloxin method

Use Zenker or formalin fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in Harris's hematoxylin (p. 548), five to ten minutes depending on the tissue. Rinse in two changes of distilled water, two minutes in each. Wash in running tap water until deep blue, thirty to sixty minutes. Pass through 60-per-cent and 80-per-cent alcohol. Stain in 0.5-per-cent phloxin in 90-per-cent alcohol, one minute. Dehydrate in 95-per-cent alcohol, one minute; two changes of absolute alcohol, two minutes in each. Clear in two changes of xylol, two minutes in each and mount in neutral xylol-dammar (p. 548).

Nuclei stain blue. Cytoplasm is red. Muscle, collagen, and elastic tissue are various shades of red and pink.

Mallory's phloxin and methylene blue stain (8b)

Methylene blue solution

Methylene blue	1 gram
Borax	1 gram
Water, distilled	100 ml.

Azure II solution

Azure II	1 gram
Water, distilled	100 ml.

For use, combine 5 ml. of the methylene blue and the azure-II solutions and add 90 ml. of distilled water.

Procedure.—Use Zenker fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in 2.5-per-cent aqueous phloxin overnight at room temperature. Wash in tap water. Stain in methylene blue and azure II solution, ten to twenty minutes, pouring the solution on and off several times. Wash in tap water. Differentiate and dehydrate in 95-per-cent alcohol to which has been added a few drops of 10-per-cent colophony (rosin) in absolute alcohol, keeping sections in constant motion so that decolorization will be uniform. Control the result with the microscope. When the pink color has returned to the section and the nuclei are still blue, finish the dehydration quickly with absolute alcohol. Clear in two changes of xylol and mount in neutral xylol-dammar (p. 548).

The cell nuclei stain blue; cytoplasm, collagen, connective tissue, and muscle, pink; red blood corpuscles, bright red.

Masson's light green trichrome method—Foot's modification (10c, 29)

Regaud's iron hematoxylin

Hematoxylin	10 grams
Alcohol, 95-per-cent	100 ml.
Glycerol	100 ml.
Water, distilled	1000 ml.

Dissolve the hematoxylin in the alcohol with gentle warming. Add the other constituents.

Picric acid alcoholic solution

Picric acid, saturated solution in 95-per-cent alcohol	2 parts
Alcohol, 95-per-cent	1 part

Ponceau acid fuchsin solutions

I. Make a 1-per-cent solution of ponceau de xylidine (Krall) in 1-per-cent acetic acid.

II. Make a 1-per-cent acid fuchsin solution in 1-per-cent acetic acid.

Combine 2 parts of solution I with 1 part of solution II.

Light green solution

Light green	2 grams
Water, distilled	100 ml.
Acetic acid, glacial	2 ml.

Boil the light green in the distilled water and add the acetic acid. Cool and filter.

Procedure.—Use Bouin fixation. Cut paraffin sections. Remove the paraffin in the usual way (p. 543).

Place in 5-per-cent aqueous solution of iron alum (p. 36) heated to 45°–50°C., five minutes. Wash in running tap water, two to five minutes. Stain in Regaud's iron hematoxylin heated to 45°–50°C., eight to ten minutes. Differentiate in alcoholic solution of picric acid until the nuclei alone remain colored. Control the result with the microscope. Wash in running tap water, thirty minutes. Stain in the ponceau-acid fuchsin mixture, five minutes. Mordant in 1-per-cent aqueous solution of phosphomolybdic acid, five minutes. Rinse in distilled water. Stain in the light green solution, five minutes. Rinse in distilled water. Place in 1-per-cent acetic acid, two minutes. Run quickly through tap water and dehydrate in ascending grades of alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Nuclei stain black or deep violet. Cytoplasm is varying shades of red. Connective tissue and mucus stain green.

METHODS FOR THE DEMONSTRATION OF MICROORGANISMS

Brown-Brenn method (30) (for differentiation of Gram-positive and Gram-negative bacteria)

Crystal violet solution

Sodium bicarbonate, 5-per-cent aqueous solution containing 0.5 per cent phenol as preservative	5 drops
Crystal violet, 1-per-cent aqueous solution	0.75 ml.

Mix in a small vial immediately before use.

Procedure.—Use Zenker fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in freshly filtered Harris's hematoxylin (p. 548), two to five minutes. Wash in distilled water, one minute, and in running tap water until blue, ten minutes. Pour the crystal violet solution on the slide and stain, two minutes. Wash quickly in tap water. Treat with Lugol's iodine solution (p. 37), one minute. Wash in tap water; blot. Decolorize in 1 part of ether and 3 parts of acetone, dropping the mixture on the slide until no more color can be removed. Dip in water for a moment. Stain with basic fuchsin (0.1 ml. saturated alcoholic solution per 100 ml. distilled water), five minutes. Wash in tap water; blot but do not allow to dry. Pass through acetone. Decolorize and differentiate by dropping over the section a solution of 0.1 gram picric acid in 100 ml. acetone until the section becomes a yellowish pink. This is the most critical step and should be performed by holding the slide over a white dish. Most of the fuchsin should be removed from the tissue but the Gram-negative bacteria should remain red. Dehydrate and clear in acetone, in equal parts of acetone and xylol, and in xylol. Mount in neutral xylol-dammar (p. 548).

The cell nuclei stain dark reddish brown; Gram-positive bacteria, deep violet or almost black; Gram-negative bacteria, bright red. Cytoplasm is yellow.

Giemsa's stain—Wolbach's modification (31–33) (for microorganisms)

Staining solution

Giemsa's stain, commercial	2.5 ml.
Alcohol, methyl	3 ml.
Water, distilled (to which 2–4 drops of 0.5-per-cent aqueous solution of sodium bicarbonate have been added)	100 ml.

Prepare immediately before use.

Procedure.—Use Zenker fixation. Cut paraffin sections, preferably 4μ. Remove paraffin in the usual manner (p. 543).

Place in 0.5-per-cent sodium hyposulfite, fifteen minutes; wash in running tap water, ten minutes. Rinse in distilled water, one to two minutes. Pour the stain over the slides; change in thirty minutes; again in one hour; and leave in the last mixture, twelve to eighteen hours. Place in distilled water. Differentiate in 95-per-cent alcohol to which a few drops of 10-per-cent colophony (rosin) in absolute alcohol has been added. Control the stain with the microscope until proper differentiation is obtained. Dehydrate rapidly in absolute alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

The results are comparable to those obtained with Mallory's phloxin and methylene blue stain (p. 550) except that the colors are somewhat less intense.

Gram-Weigert method (34, 35, 8c) (for Gram-positive bacteria)

Zenker fixation is preferable. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain lightly in Harris's hematoxylin (p. 548), two to three minutes; wash in running tap water. Stain in 2.5-per-cent aqueous solution of phloxin, one to five minutes; wash in water. Stain in aniline crystal violet (p. 547), thirty to sixty minutes; wash in tap water. Treat with Lugol's iodine solution (p. 37), one to two minutes; wash in tap water. Blot with filter paper, decolorize and clear in several changes of equal parts of aniline and xylol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Gram-positive microorganisms stain deep violet. Connective tissue is red; nuclei, blue to violet.

Heidenhain's iron hematoxylin method (36) (for Endamoeba histolytica)

Use Schaudinn's fluid or Zenker or mercuric chloride-alcohol fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Immerse sections, keeping them in an upright position, in 4-per-cent aqueous solution of iron alum (p. 36), twelve to twenty-four hours. Rinse in tap water several minutes. Stain in 0.5-per-cent Heidenhain's iron hematoxylin (p. 36) which is at least four to seven weeks old, until black, twelve to twenty-four hours. Rinse in distilled water. Differentiate in 2-per-cent aqueous solution of iron alum until the tissues are a pale gray and the nuclei, black. Control the result with the microscope. It is helpful to alternate from the alum solution to tap water several times during the process of differentiation. Rinse in distilled water. Dehydrate in 60-, 90-, and 95-per-cent alcohol, two minutes in each; two changes of absolute alcohol, one minute in each. Clear in two changes of xylol, two minutes in each. Mount in neutral xylol-dammar (p. 548).

Cytoplasm is grayish while the structures of the nucleus are jet black. The caryosome appears as a small black dot in the center of the nucleus.

Mallory's method (37, 8d) (for Actinomyces)

Use formalin fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in Harris's hematoxylin (p. 548), three to five minutes. Wash in water. Stain in 2.5-per-cent aqueous solution of phloxin, fifteen minutes in the incubator at from 54° to 55°C. Wash in tap water. Stain in aniline crystal violet (p. 547), five to fifteen minutes. Wash in tap water. Treat with Gram's iodine (p. 36), one minute. Wash in tap water; blot. Differentiate in several changes of aniline until no more color is removed. Clear in several changes of xylol and mount in neutral xylol-dammar (p. 548).

Branched microorganisms stain blue; clubs, pink to red.

Nicolle's carbolthionine solution (38) (for microorganisms)

Carbolthionine solution

Thionin	1 gram
Phenol	1 gram
Alcohol, 90-per-cent	10 ml.
Water, distilled	100 ml.

Procedure.—Zenker fixation is preferable. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in carbolthionine solution five minutes. Rinse in distilled water; blot dry. Dehydrate rapidly in alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Bacteria stain deep blue; cells and nuclei, lighter blue.

Nyka's method (39) (for Rickettsia)

Basic fuchsin, 5-per-cent solution

Basic fuchsin	5 grams
Alcohol, 90-per-cent	100 ml.

Procedure.—Fix tissue, about 0.5 by 0.5 by 0.2 cm., in Regaud's (Müller's) fluid, p. 549. Cut paraffin sections 1-4μ. Remove paraffin in the usual manner (p. 543).

Stain in 5-per-cent basic fuchsin solution, five to ten minutes. Rinse rapidly in tap water. Differentiate in 90-per-cent alcohol, one to two minutes. Stain in a 1:10,000 dilution of aqueous methyl violet, one to three minutes. Rinse in tap water. Differentiate thoroughly in a 1:1500 dilution of acetic acid. Dehydrate in absolute alcohol, clear in xylol, and mount in neutral xylol-dammar.

Cytoplasm and red blood cells stain pale yellow or pink; rickettsiae and nuclei, violet. The rickettsiae are seen scattered among the cells or in clumps inside the cells.

Pinkerton's method—modified (21, 8e) (for Rickettsia)

Fix tissue not more than 5 mm. in thickness in Regaud's fluid. Wash in running tap water, twenty-four hours, and dehydrate with dioxane in the usual manner. Embed in paraffin and cut sections 4μ. Remove paraffin in the usual manner (p. 543). Pass through descending percentages of alcohol and then into distilled water, neutral in reaction. Stain in Giemsa's solution (p. 552). Differentiate in 95-per-cent alcohol until the red blood cells turn from green to pink; the rickettsiae stain bluish-purple, and the cytoplasm, blue. Dehydrate in absolute alcohol, clear in xylol, and mount in neutral xylol-dammar.

For staining *Dermacentroxenus rickettsi* (40): Fix tissues in Regaud's solution and prepare sections as described in the previous paragraph; dehydrate in the usual way. Stain in 1-per-cent aqueous methylene blue overnight, decolorize in 95 per-cent alcohol, counterstain in 0.25-per-cent aqueous basic fuchsin for thirty minutes. Decolorize rapidly in 0.5-per-cent citric acid, three seconds. Wash lightly in distilled water. Counterstain in 1-per-cent aqueous methylene blue, five seconds. Differentiate in 95-per-cent and absolute alcohol, clear in xylol, and mount in neutral xylol-dammar.

The rickettsiae stain a deep blue against a background that is partly red and partly blue.

Tanner's fluorescence method (41) (for acid-fast bacilli)

Auramine stain

Solution I

Phenol	3 grams
Water, distilled	87 ml.

Solution II

Auramine-O	0.1 gram
Alcohol, 95-per-cent	10 ml.

Add solution II to solution I.

Acid alcohol

Sodium chloride	0.5 gram
Hydrochloric acid (sp. gr. 1.19)	1 ml.
Alcohol, 70-per-cent	100 ml.

Procedure.—Use formalin fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in auramine solution, five minutes. Wash in tap water. Decolorize with acid alcohol, twenty minutes. Wash in tap water. Place again in acid alcohol, twenty minutes. Wash in tap water. Counterstain with a 0.1-per-cent aqueous methylene blue, one minute. Wash and dry the sections and examine with the fluorescence microscope without the use of a cover glass.

The bacilli are seen as bright yellow rods on a black or dark-blue background.

Warthin-Starry method—modified (42) (for Treponema pallidum)

Acidulated water pH 4.4

Citric acid, 1-per-cent aqueous solution	15 drops
Water, distilled	1000 ml.

Developing solution

Gelatin, 5-per-cent aqueous solution (45°C.)	15 ml.
Silver nitrate, 2-per-cent aqueous solution	3 ml.
Hydroquinone, 5-per-cent aqueous solution	1 ml.

Use acidulated water, pH 4.4, in the preparation of the silver nitrate solution. The gelatin should be kept at 45°C. Mix the gelatin and silver nitrate solutions well and add the hydroquinone. Mix thoroughly. It is essential that the developing solution be prepared fresh each time immediately before use.

Procedure.—Use formalin fixation. Cut paraffin sections and mount on No. 1

cover glasses without the albumen fixative. Dry in the incubator at 54°–55°C., one to twenty-four hours. Remove the paraffin in the usual manner (p. 543).

Rinse in acidulated water, pH 4.4. Rinse in 0.5-per-cent silver nitrate solution, pH 4.4. Impregnate at 37°–38°C. in the dark, one hour, by placing the cover glasses on edge, the section side resting against the wall of a perfectly clean, square dish filled with sufficient silver nitrate solution to cover the section by capillary action. Heat the developing solution to 45°–50°C. Place the slides (section side up) in a Petri plate and pour it over them. Allow the sections to remain in the developer until a brownish-black or golden-yellow color appears, three to eight minutes, depending on the type of tissue. Stop the process by rinsing sections in distilled water at 50°C. Place in 5-per-cent sodium thiosulfate, three to five minutes; wash in distilled water. Dehydrate through 95-per-cent alcohol, two minutes; two changes of absolute alcohol, two minutes each; two changes of xylol, two minutes each. Mount in neutral xylol-dammar (p. 548).

Treponema pallidum stains varying shades of black against a golden-yellow or brownish background.

Ziehl-Neelsen method (43, 44)—modified (for acid-fast bacilli)

Use Zenker or formalin fixation, preferably Zenker. Cut paraffin sections, approximately 10μ. Remove paraffin in the usual manner (p. 543).

Transfer to 0.5-per-cent sodium bicarbonate, three to five minutes. Cover with carbolfuchsin (p. 43) and steam gently, five to seven minutes. Wash in tap water. Decolorize with acid alcohol (p. 546) for thirty seconds until no more red is extracted. Wash in tap water. Counterstain with alkaline methylene blue (p. 37), diluted 1:10, one minute. Wash in tap water; dehydrate in 95-per-cent alcohol, one minute; two changes of absolute alcohol, 2 minutes each. Clear in xylol, one minute, and mount in neutral xylol-dammar (p. 548).

Better results may be obtained frequently by a frozen-section technic particularly when tissue is formalin fixed.

Cut frozen sections, 16–18μ. Float into a Stender dish of distilled water. Transfer with a glass rod to a 1-per-cent sodium bicarbonate solution, two to three minutes. Stain in 4-per-cent carbolfuchsin, thirty minutes at room temperature. Wash in tap water. Decolorize in acid alcohol for about thirty seconds until the sections are a faint violet-pink color. Wash in tap water. Counterstain in alkaline methylene blue, diluted 1:10, two minutes. Dehydrate quickly in 95-per-cent alcohol and two changes of absolute alcohol. Clear in xylol; mount in neutral xylol-dammar.

Acid-fast bacilli stain deep red.

METHODS FOR STAINING CONNECTIVE TISSUE

Foot's method (45, 46) (for reticulum)

Silver ammonium carbonate solution

Silver nitrate, 10-per-cent aqueous solution	10 ml.
Lithium carbonate, saturated aqueous solution (1.25–1.50 grams) per 100 ml. distilled water	10 ml.

Use distilled water in the preparation of these solutions. Combine the silver nitrate and lithium carbonate solutions and allow the precipitate to settle. Wash it three times with 50 ml. of distilled water. Add 25 ml. of fresh distilled water to the washed precipitate. While the container is shaken continuously, add, drop by drop, 8–15 drops of 26–28-per-cent ammonium hydroxide until the precipitate turns a grayish brown and is nearly dissolved. Extreme care is needed not to pass the end point. A few granules of the precipitate should remain undissolved. Dilute to 100 ml. with distilled water. Prepare each time sections are to be stained.

Procedure.—Use Zenker fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Treat with a 0.5-per-cent iodine solution in 95-per-cent alcohol, five minutes. Wash in tap water and bleach in 5-per-cent aqueous sodium thiosulfate solution. Wash in tap water. Treat with 0.25-per-cent aqueous potassium permanganate, five minutes. Rinse in tap water. Treat with 5-per-cent aqueous oxalic-acid solution, ten minutes. Wash in tap water followed by distilled water. Immerse in silver ammonium carbonate solution heated to 50°C. to remove excess ammonia and place in a 37°C.-incubator, fifteen to twenty minutes. Wash quickly in distilled water. Reduce in 20-per-cent neutral formalin, two to five minutes. Rinse well in tap water. Treat in an aqueous solution of gold chloride, diluted 1:500, two to five minutes. Wash and fix in 5-per-cent aqueous sodium-thiosulfate solution, two to five minutes. Wash well and stain with Harris's hematoxylin (p. 548), three to five minutes. Rinse in distilled water. Wash in running tap water until blue, ten minutes. Stain in Van Gieson's stain (p. 559), forty-five seconds. Dehydrate in 95-per-cent alcohol followed by absolute alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Coarse collagenous fibrils stain red. Reticulum is black to dark violet; nuclei are black, blue to brownish; cytoplasm is grayish yellow. Muscle fibers and elastic tissue stain light yellow.

Mallory's aniline blue stain (47) (for collagen)

Aniline blue stain

Aniline blue, water soluble	0.5 gram
Orange G (dye content 85–90)	2 grams
Phosphotungstic acid	1 gram
Water, distilled	100 ml.

Procedure.—Use Zenker fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in 0.25-per-cent aqueous solution of acid fuchsin, thirty minutes. Drain and stain in aniline blue, one to twenty-four hours or more at room temperature or one hour in the incubator at 54°–55°C. Transfer to 95-per-cent alcohol, three changes, until no more color is removed. Dehydrate in absolute alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Collagen stains varying shades of blue. Nuclei, fibroglia, fibrin, myoglia are red; red blood cells, yellow. Elastic tissue stains pale pink or yellow.

Mallory's aniline blue—Heidenhain's azocarmine stain—McGregor's modification (47–49) (for basement membrane of the glomerulus)

Azocarmine stain

Azocarmine G, 1-per-cent	1 gram
Water, distilled	100 ml.

Bring to a boil, cool to room temperature, and filter through coarse filter paper. Add 1 ml. of glacial acetic acid.

Aniline blue stain

Aniline blue, water soluble	0.5 gram
Orange G (dye content 85–90)	2 grams
Acetic acid, glacial	8 ml.
Water, distilled	100 ml.

Boil, cool, and filter. For staining, take an equal amount of stock solution and distilled water.

Aniline alcohol

Aniline	1 ml.
Alcohol, 95-per-cent	100 ml.

Procedure.—Use Zenker fixation. Cut paraffin sections. Remove the paraffin in the usual manner (p. 543).

Stain in azocarmine in the incubator at from 54° to 55°C., thirty to forty minutes. Differentiate in aniline alcohol until the nuclei are red and the cytoplasm is pink. Control the result with the microscope. Remove aniline from the sections with acid alcohol, one minute; treat in 5-per-cent phosphotungstic acid, three hours. Wash quickly in tap water. Stain in aniline blue, three to six hours. Wash in tap water. Dehydrate in absolute alcohol and control the stain with a microscope. Clear in two changes of xylol and mount in neutral xylol-dammar (p. 548).

The nuclei stain orange red; connective tissue, fibrin, and reticulum, blue. Cytoplasm is pink.

Mallory's phosphotungstic acid-hematoxylin stain (50) (for fibrils)

Phosphotungstic acid-hematoxylin solution

Hematoxylin	0.1 gram
Phosphotungstic acid	2 grams
Potassium permanganate, 0.25-per-cent aqueous solution	10 ml.
Water, distilled	100 ml.

Dissolve the hematoxylin in a portion of the water with the aid of heat. Cool and add the potassium permanganate solution. Dissolve the acid in the remainder of the water and add to the dissolved hematoxylin. The solution requires several weeks to ripen.

Procedure.—Use Zenker fixation. Cut paraffin sections. Remove the paraffin in the usual manner (p. 543).

Place sections in a 0.25-per-cent solution of potassium permanganate, five to ten minutes. Wash in tap water. Place in 5-per-cent aqueous oxalic acid solution, ten to twenty minutes. Wash thoroughly in several changes of tap water. Stain in phosphotungstic acid hematoxylin, twelve to twenty-four hours. Transfer directly to 95-per-cent alcohol and dehydrate rapidly in absolute alcohol, two changes. Clear in xylol and mount in neutral xylol-dammar (p. 548).

The nuclei, neuroglia, fibroglia, and myoglia fibrils and fibrin stain blue; collagen and the ground substances of cartilage and bone, varying shades of yellow to brownish red. Course elastic fibrils are sometimes colored a purplish tint.

Taenzer-Unna Orcein method—modified (51, 52) (for elastic fibers)

Orcein solution

Orcein	0.5 gram
Alcohol, absolute	40 ml.
Hydrochloric acid (sp. gr. 1.19)	20 drops
Water, distilled	20 ml.

Acid-alcohol mixture

Hydrochloric acid (sp. gr. 1.19)	0.1 ml.
Alcohol, 95-per-cent	20 ml.
Water, distilled	5 ml.

Procedure.—Formalin fixation is preferable. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in the orcein solution, twelve to twenty-four hours. Differentiate in the acid-alcohol mixture. Rinse in tap water. Dehydrate in 95-per-cent alcohol; two changes of absolute alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

The elastic fibers are stained dark reddish brown; the nuclei, light reddish brown. A slight tinging with aniline blue causes the elastic elements to stand out better against the delicate red connective tissue.

Van Gieson's stain (53, 54) (for connective tissue)

Picro-acid fuchsin solution

Picric acid, saturated aqueous solution (about 1.25 per cent)	100 ml.
Acid fuchsin, 1-per-cent aqueous solution	5–15 ml.

To determine the exact amount of fuchsin, stain a section containing collagen.

Procedure.—Use either formalin or Zenker fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain deeply with Harris's hematoxylin (p. 548). Rinse in distilled water. Wash in running tap water until blue, twenty to thirty minutes. Stain with picro-acid fuchsin solution, three to seven minutes. Transfer to 95-per-cent alcohol. Dehydrate in absolute alcohol, one minute. Clear in two changes of xylol, one minute in each. Mount in neutral xylol-dammar (p. 548).

Connective tissue stains deep red; muscle, yellow.

Verhoeff's stain (55) (for elastic tissue)

Hematoxylin solution

Hematoxylin	1 gram
Alcohol, absolute	20 ml.

Dissolve by heating and filter.

Add the following in the order given:

Ferric chloride, $FeCl_3 \cdot 6H_2O$, 10-per-cent aqueous solution	8 ml.
Iodine solution (Lugol, p. 37)	8 ml.

Procedure.—Use formalin or Zenker fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543), except that if the tissue is fixed in Zenker's fluid, do not treat with iodine solution before staining.

Stain in a freshly prepared solution, fifteen minutes or until perfectly black. Differentiate quickly in a 2-per-cent aqueous solution of ferric chloride. Use the microscope to control the degree of differentiation. Wash in tap water and in 95-per-cent alcohol to remove the iodine. Wash in tap water. Counterstain with 0.5-per-cent phloxin in 90-per-cent alcohol, one minute. Dehydrate in 95-per-cent alcohol and two changes of absolute alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Elastic tissues stain black; connective tissue, fibroglia, myoglia and neuroglia, red to pink.

METHODS FOR STAINING HEMATOPOIETIC TISSUE

Custer's method (56) (for bone marrow)

Solution I

Eosin Y	1 gram
Water, double distilled	1 liter

Solution II

Azure II	1 gram
Water, double distilled	1 liter

Just before use, add 20 ml. of solution I and 10 ml. of solution II to 8–10 ml. of double distilled water. Filter.

Procedure.—After decalcifying in Wagoner's solution (p. 549), wash gently in running tap water, twelve to twenty-four hours, dehydrate with dioxane, and embed in paraffin in the usual manner. Remove paraffin in the usual manner (p. 543). Cut sections 4μ, dehydrate in xylol and treat the same as for Zenker fixation. Place the slides in a Coplin jar filled with staining fluid and cover. Allow to stand overnight. Differentiate in two changes of 95-per-cent alcohol until the erythrocytes are red and the nuclei are sharp and brilliant blue. Control the results with a microscope. Transfer to two changes of absolute alcohol and three changes of xylol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Maximow's azure II eosin and hematoxylin stain (57) (for hematopoietic tissue)

Solution I

Eosin, water soluble, yellowish W. G	0.5 gram
Water, double distilled	500 ml.

Solution II

Azure II	0.5 gram
Water, double distilled	500 ml.

Add 10–12 ml. of solution I to 100 ml. distilled water, then add 9–10 ml. of solution II.

Procedure.—Use Zenker-formol fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in 1 drop of Harris's hematoxylin (p. 548) to 100 ml. of double distilled water, twenty-four hours. Wash in several changes of freshly distilled water, twenty-four hours. Chromatin should be blue; cytoplasm, colorless or light gray. Stain the slides in an upright position in azure II eosin solution, twenty-four hours. Differentiate and dehydrate in 95-per-cent alcohol, one minute; two changes of absolute alcohol, one minute each. A few drops of 10-per-cent colophony (rosin) in absolute alcohol are helpful. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Chromatin stains dark blue; nucleoli are purple. Leucocytic granules are differentially stained as in Giemsa's (p. 552) or Wright's method (p. 42).

Weigert's stain (35, 8f) (for fibrin)

Lithium carmine solution

Carmine	2.5–5 grams
Lithium carbonate, saturated aqueous solution (1.25–1.50 grams per 100 ml. distilled water)	100 ml.
Thymol	1 gram

Dissolve the carmine in lithium carbonate and boil for fifteen minutes. Cool and add thymol. Filter before use.

Crystal violet solution

Alcohol, absolute	33 ml.
Aniline	9 ml.
Crystal violet, in excess	

Just before use, combine 3 ml. of the crystal violet solution and 27 ml. of a saturated aqueous solution of crystal violet (approximately 1.68 per cent).

Procedure.—Alcohol fixation is preferable. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Stain in lithium carmine, two to five minutes. Differentiate in acid alcohol (p. 546). Wash thoroughly in tap water. Stain in crystal violet solution, five to ten minutes. Pour over the sections Gram's iodine solution (p. 36) and allow to remain, ten minutes. Blot. Differentiate in equal parts of aniline and xylol by pouring the mixture over the sections until no more color is removed. Blot. Wash in several changes of xylol and mount in neutral xylol-dammar (p. 548).

Fibrin stains blue to blue black.

HISTOCHEMICAL METHODS

Berlin blue method (58) (for iron)

Potassium ferrocyanide solution

Potassium ferrocyanide, $K_4Fe(CN)_6 \cdot 3H_2O$, 2-per-cent aqueous solution freshly prepared	1 part
Hydrochloric acid (sp. gr. 1.19), 1-per-cent aqueous solution (by volume)	3 parts

Procedure.—The knife should be perfectly clean and free from rust. Use formalin or alcohol (absolute or 95-per-cent) fixation. Cut frozen or paraffin sections. If paraffin sections are made, remove paraffin in the usual manner (p. 543).

Stain in potassium ferrocyanide heated to steaming in a glass beaker, one minute. Wash immediately and thoroughly in several changes of tap water. Counterstain in 0.1-per-cent basic fuchsin in 50-per-cent alcohol, twenty to thirty minutes. Differentiate and dehydrate in 95-per-cent alcohol, followed by absolute alcohol. Clear in xylol and mount in neutral xylol-dammar (p. 548).

Hemosiderin stains blue.

Best's carmine stain (59, 60) (for glycogen)

Carmine solution

Carminum rubrum optimum	2 grams
Potassium carbonate, $2K_2CO_3 \cdot 3H_2O$	1 gram
Potassium chloride	5 grams
Water, distilled	60 ml.

Boil gently until the color darkens. Allow to cool. Add 20 ml. of concentrated ammonium hydroxide (26–28 per cent NH_3 by weight). Ripen for twenty-four hours. This solution keeps for approximately one month.

Diluted staining solution

Stock carmine solution	10 ml.
Ammonium hydroxide (26–28 per cent NH_3 by weight)	15 ml.
Alcohol, methyl	30 ml.

Stir thoroughly and use at once. Do not filter.

Procedure.—Glycogen is readily soluble in water. This fact must be kept in mind throughout all procedures undertaken for fixation and staining. While celloidin impregnation is usually recommended, paraffin sections can be made if especially treated.

Fix in absolute alcohol. Embed in paraffin in the usual manner and cut sections 6–8μ. Float in 95-per-cent alcohol which has been heated to 40°C. on an electric plate and attach to the slides with Mayer's egg albumen fixative (p. 548). Remove paraffin in the usual manner by xylol (p. 543) and leave in absolute alcohol. Transfer sections to a mixture of equal parts of absolute alcohol and ether containing about 0.5 per cent celloidin in a Coplin jar. Cover and allow sections to remain for two or more hours. Remove and allow ether to evaporate. Transfer to 80-per-cent alcohol, ten minutes; distilled water, one minute. Stain in Harris's hematoxylin (p. 548), three to five minutes. Wash in running tap water until blue. Stain in freshly prepared diluted Best's carmine solution, twenty minutes. (As a control, stain a section of liver.) Rinse in three changes of methyl alcohol. Dehydrate in two changes of absolute alcohol. Clear in xylol and mount in neutral xylol-dammar.

Glycogen stains a brilliant vermilion-red color.

Congo red—Bennhold's method (61) (for amyloid)

Use formalin fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543). Place sections in distilled water, one to two minutes. Stain in a 1-per-cent aqueous solution of congo red, fifteen to thirty minutes. Place in a saturated aqueous solution of lithium carbonate, fifteen minutes. Decolorize in 80-per-cent alcohol. Wash in tap water, fifteen minutes. Counterstain with Harris's hematoxylin (p. 548). Wash in tap water. Dehydrate in 95-per-cent and absolute alcohol. Clear in xylol and mount in neutral xylol-dammar.

Amyloid stains red.

Congo red—Thomlinson's method (61, 62) (for amyloid)

Use formalin fixation. Cut frozen sections. Stain in Harris's hematoxylin (p. 548), fifteen to thirty seconds. Wash in tap water. Intensify the blue in lithium carbonate, one to two minutes. Transfer directly from the lithium carbonate to a 1-per-cent aqueous solution of congo red and leave five minutes. Wash thoroughly in tap water. Mount in glycerol.

Amyloid stains red.

Mayer's stain (for amyloid)

Use formalin or Zenker fixation. Cut paraffin sections.

Remove them directly from the knife to a 0.5-per-cent aqueous solution crystal violet warmed to 40°C., ten minutes. Wash in tap water and differentiate in a 1-per-cent solution of acetic acid, ten to fifteen minutes. Wash thoroughly in tap water. Transfer to a half saturated aqueous solution of aluminum ammonium sulfate, $AlNH_4(SO_4)_2 \cdot 12H_2O$ (7.5 per cent). Wash in tap water. Float sections onto slides and allow water to evaporate. Remove paraffin and clear with xylol. Mount in neutral xylol-dammar (p. 548).

Amyloid stains a reddish violet.

Mucicarmine with hematoxylin and metanil yellow—adapted from Masson (63, 25b) (for mucin)

Mayer's mucicarmine stain (64)

Carmine	1 gram
Aluminum chloride, anhydrous	0.5 gram
Water, distilled	2 ml.

Combine and heat slowly over a low flame, stirring constantly, until the mixture dissolves and becomes dark. Continue to stir and gradually add 100 ml. of 50-per-cent alcohol. Let stand overnight and filter. The stain keeps well.

Before use, dilute 1:10 with distilled water.

Metanil yellow stain

Metanil yellow	0.5 gram
Acetic acid, 0.2-per-cent aqueous solution	100 ml.

Store in a brown bottle. The stain may be used repeatedly.

Procedure.—Use formalin fixation. Cut paraffin sections. Remove paraffin in the usual manner (p. 543).

Wash in distilled water. Stain in Weigert's iron hematoxylin (p. 549), one minute. Wash in distilled water. Stain in metanil yellow, thirty seconds. Wash in distilled water. Stain in mucicarmine, forty-five minutes. Wash quickly in distilled water. Dehydrate rapidly in absolute alcohol, clear in xylol, and mount in neutral xylol-dammar.

Mucin stains bright red.

Scharlach R (Sudan IV) stain (65, 66) (for fat)

Scharlach R solution

Alcohol, 70-per-cent	50 ml.
Acetone	50 ml.
Scharlach R, sufficient for a saturated solution	0.1–0.2 gram

Procedure.—Use formalin fixation. Cut frozen sections 10μ. Dip for an instant in 70-per-cent alcohol.

Stain in tightly stoppered wide-mouthed bottles to prevent evaporation and formation of precipitate, five minutes. Wash quickly in 70-per-cent alcohol. Transfer to distilled water. Counterstain with Harris's hematoxylin (p. 548). Place in 1-per-cent acetic acid, three to five minutes. Wash in tap water. Mount in glycerol.

Fat stains orange to red.

MUSEUM

PREPARATION OF GROSS SPECIMENS

When a specimen of tissue is prepared for the museum, assign a museum accession number to it. Sew on it a fiber tag deeply punched with the number for identification during the procedures of fixation. Examine the tissue and record pertinent data from the history, together with a brief description of the material in the gross. The specimen may then be photographed if this appears warranted. Cut an adequate number of blocks to illustrate the tissue changes that have occurred and make sections for histologic study. Trim the specimen so that the interesting features will be shown to advantage. The final dissection, however, is made at the time of mounting. Attach to a cork float and suspend in about twelve to fifteen times its volume of Kaiserling-I solution, Lundquist and Robertson's modification (p. 548). Pass illuminating gas into the jar to preserve the original color (67, 68).

For this purpose, a museum jar securely fitted with a wooden cover that is perforated with two holes for tubing can be used. The rim of the cover is coated with a mixture of wax and vaseline to prevent the escape of gas. A curved glass tube reaching almost to the bottom of the jar is passed through one of the holes in the cover; a rubber tube connects this with the gas outlet. Another tube is passed through the second hole and connected with a rubber tube that opens into a fume hood.

Regulate the pressure so that the gas just bubbles through the solution and continue the operation for about one-half hour. Transfer the specimen to a new Kaiserling-I solution, and allow to remain until fixed. The time required depends upon the kind of tissue, as well as the size of the specimen. If the specimen is large, make several deep incisions to permit more thorough fixation. Make these incisions through parts of the tissue where as little distortion as possible will result when the preparation of the specimen is completed. When entirely fixed, transfer the specimen to running water and wash to remove all traces of formalin. Trim further, remove excess tissue and fat, and smooth the edges. The second solution which Kaiserling used has been found to be unnecessary. To remove the water, place in Kaiserling-III solution, Lundquist and Robertson's modification, for twenty-four hours or longer depending upon the size of the specimen. Transfer to a second Kaiserling-III solution that has been filtered and leave until ready for mounting.

MOUNTING

Specimens are mounted in the McGill type of rectangular glass jar, in the thin cells described by Davis, and under clock glasses. For specia purposes, other mounts may be used.

Glass-Jar Method.—Select a jar slightly larger than the specimen. Construct a support from glass rods of appropriate size by fusing them together in such a manner as to conform to the shape of the specimen and to fit the jar exactly. Pyrex rod may be used to advantage; but the same kind of glass should be throughout in any given frame. The completed frame should be annealed, in a yellow gas flame for a few minutes if flint glass is used, to reduce the stresses resulting from fusing. Anchor the specimen to the frame with linen-coated thread and replace the fiber tag with a paraffined paper tag bearing the number of the specimen for permanent identification. Place in a clean jar and add fresh filtered Kaiserling-III solution to within approximately 3 cm. of the top. Seal the jar with hot asphalt cement (p. 546) according to Muir and Judah (13) by means of an electric applicator (69). Fill the applicator with the cement and turn on the current. Heat until the cement flows freely. While it is melting, clean the rim of the jar and of the cover with soap and water and absolute alcohol. Heat these surfaces with a flame until hot to the touch. Draw the tip of the applicator along the top of the jar near the outer edge. The amount of cement required can be regulated by the speed at which the applicator is moved. Weight the lid down on the jar until the cement is cool. Add additional filtered Kaiserling-III solution through the small hole in the cover until the jar is filled to within approximately 0.5 cm. of the top. Insert a cork in the hole and cover with asphalt.

Davis's Thin-Cell Method (70) **Slightly Modified.**—This is an economical method that displays sections of tissue admirably. Use glass photographic plates (5 by 7 inches) in the preparation of the cell. Cut two strips of brass about $\frac{1}{4}$ inch in width and $16\frac{3}{4}$ inches in length. File them $6\frac{1}{4}$ inches from each end to permit them to be bent at an angle of 90°. Make another strip the same width and $4\frac{3}{8}$ inches long with a hole bored approximately $\frac{1}{2}$ inch from each end. Cut a piece of battleship linoleum

$4\frac{1}{4}$ inches by 8 inches. Bend the longer piece of brass around one end of the linoleum. Place the glass plates on each side so that the brass strip will be $\frac{3}{8}$ inch from the edge. Hold the cell together with a large paper clip on each side. Fill one groove about one-half full with asphalt cement, using the electric applicator. When hard—after a few minutes—apply cement to the other two grooves. Add to the first layer, a second layer to fill the grooves. Remove the linoleum.

Section the tissue fixed and preserved in the Kaiserling solutions in the usual manner. Slice it on a board equipped with $\frac{3}{16}$ inch square brass rails provided with lugs that fit into holes in the board so that the rails may be adjusted to fit the specimen, thus facilitating the cutting of a piece uniform in thickness. The specimen is then sectioned by drawing a long sharp brain knife diagonally across the brass rails. It is important to complete the cutting with one stroke in order to obtain a smooth surface. Choose the pieces that are perfectly plain with parallel sides and show the lesion best for display. Attach a paraffined paper tag bearing the number of the specimen for permanent identification. Wash the tissue in running water and place in two changes of a 50-per-cent cane-sugar solution saturated with thymol, six hours in each. Fill the cell about one-half to two-thirds full of the cane-sugar preservative to prevent growth of mold. Insert the tissue and displace the air bubbles. Fit the short piece of brass with the hole in each end in place and cover the last groove with asphalt. When this has hardened, open the holes with a hot needle. Completely fill the cell by means of a glass cannula connected by a rubber tube to the stock bottle of mounting medium which is under slight pressure. Displace the air bubbles and seal with hot asphalt cement. The edge of the cell can be painted with Duco. If at any time the fluid in the cell becomes cloudy, open the holes. Insert in one the cannula attached to the stock bottle and displace the old fluid with new, care being taken not to allow air to enter as air bubbles are very difficult to remove from the tissue in the closed mount.

Clock Glass Method (71, 72).—Choose a clock glass that will accommodate the tissue to be mounted. Slice the specimen as described under "Davis's Thin Cell Method." Select pieces that are perfectly plain with parallel sides and show the lesion best for display. Attach a paraffined paper tag bearing the number of the specimen for permanent identification.

Prepare the clock glass by grinding the rim and polishing it so that there will be perfect adhesion when placed in apposition to the plate-glass base. While grinding, move the clock glass eccentrically on a slab of glass on which moistened Turkish emery has been placed. Continue the grinding for one to two hours or until a plain facet is formed. Polish with moistened white rouge until perfectly smooth. The grinding and polishing may be done by hand, but considerable time may be saved by the use of a motor with a suction applicator that can be attached to the convex surface of the clock glass. Place the tissue in a desiccator containing filtered Kaiserling-III solution or other suitable preservative. Exhaust the air by vacuum. Slide the clock glass under the specimen in the fluid. Select a glass base approximately $\frac{1}{8}$ in. thick and of suitable size to cover the clock glass and submerge it also in the medium. Center the clock glass and plate and press firmly together. Be sure no bubbles of air are entrapped. Remove the mount and wash the surfaces with soap and water followed by acetone. Allow it to set in a horizontal position for several hours. If the clock glass has been ground perfectly, no air bubbles should collect. Seal with marine varnish.

Special Mounts.—The method of mounting calculi is based on that of Brites (73). Insert the calculus into a tube of a diameter slightly larger than that of the specimen itself. Plug the tube with a tuft of glass wool so that when it is inverted the calculus will rest on it. Draw the base of the tube into a capillary. Evacuate the air, seal off the capillary in a flame, and embed the sealed end in a square plaster-of-Paris base.

To mount gall stones *in situ*, cut a window in the gall bladder. Place the calculi in 20-per-cent gelatin, then embed in the prepared gall bladder. Mount the specimen by the clock-glass method.

Judah's method (74), somewhat modified, is used for cleaning large bones. Remove as much of the flesh as possible and immerse the specimen in a 10-per-cent solution of potassium hydroxide for from five to ten minutes. Remove and wash in boiling water, brushing off the soft parts with a stiff nail brush. Repeat the procedure until the bone is clean. Dry, and, if grease remains, soak in gasoline until all fat is removed. Bleach in 10-per-cent hydrogen peroxide.

For small skeletal specimens, Izzard's method (75) is followed. Remove or slit the skin. Macerate the specimen in distilled water not longer than seven days at a temperature of 30°–35°C. The action is greatly facilitated if the water is changed every two days. Bones must not be allowed to float during the process. Place in boiling water and clean with a fine brush. Dry, place in benzine for two or three days to remove any grease, and dry. The disarticulated bones may be held together by nitrocellulose (20 sec. viscosity) added to methyl acetate until a heavy viscous solution is formed. Mount the completed skeleton on stainless steel wire and attach to an appropriate base.

Satisfactory methods for the permanent display of the cultural characters of bacteria are not at present available. Cultures sealed in tubes or Petri plates or exposed to illuminating gas and then to formaldehyde gas for successive 15-minute periods may be satisfactory for a limited length of time. Petri plates containing cultures that have thus been treated can be attached to glass plates similar to those used in clock-glass mounts with paraffin at a low melting point.

Somewhat more permanent mounts have been made of cultures of tubercle bacilli by the following procedure: Kill the growth by placing the open Petri plate face down on a rack in a desiccator containing a small amount of formalin. Leave for at least forty-eight hours. Prepare a clock glass in the same manner as previously described for the display of tissue. Partially fill it with a warm 20-per-cent solution of gelatin. Remove colonies with the medium and slide them face down into the glass and evacuate the air bubbles. Cover the bottom of the desiccator with formalin. Place the glass in the desiccator, cover, and allow to stand for twenty-four hours to formalinize the gelatin. After removal from the desiccator, add a few drops of water to maintain vapor tension. Place a glass plate over the clock glass and, without extraction of air, dry, and seal with marine varnish. Specimens prepared in this manner have remained in a satisfactory condition for five years.

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METHODS USED IN THE LABORATORIES FOR SANITARY AND ANALYTICAL CHEMISTRY

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METHODS USED IN THE LABORATORIES FOR SANITARY AND ANALYTICAL CHEMISTRY

INTRODUCTION

The laboratories for sanitary and analytical chemistry make bacteriologic and chemical examinations of water and sewage from the public water supply and sewage disposal systems throughout the state in connection with the control exercised by the Department of Health in the operation of these plants. Bacteriologic examinations are also made of the water supplies of camps and summer hotels whenever, in the judgment of the field inspectors, they are considered necessary to the control of sanitation. Samples of water from other sources are examined whenever in the opinion of the health officer the results are likely to be directly applicable to the prevention of disease or the protection of public health. Bacteriologic examinations of milk and cream and tests to determine pasteurization are made in cooperation with the Bureau of Milk Sanitation in its supervision of pasteurizing plants, and in other special investigations.

The work of the laboratories is not limited to these examinations. Field studies of stream pollution and of the operation of water and sewage treatment plants or of the disposal of industrial wastes are made with increasing frequency in conjunction with the engineering staff of the Division of Sanitation. Other studies are also made as the need arises and facilities permit. Researches to improve methods are undertaken; these investigations have an important application in the establishment of standards in the local approved laboratories. Short courses of instruction in laboratory methods are given in the regional training schools for waterworks and sewage-plant operators and for milk inspectors. These schools are municipal training institutes chartered by the Regents of the University of the State of New York and are conducted jointly by several interested agencies, including the faculties of the universities in which the courses are given.

In the following pages the policies that guide the relations of the sanitary and analytical chemical laboratories to outside agencies regarding the work that is undertaken, for whom it is done, and the systems of reporting are stated in detail. In general, the methods used are those of the American Public Health Association, the American Water Works Association, the Federation of Sewage Works Associations, and the Association of Official Agricultural Chemists. The procedures outlined show the adaptations that have been found most suited to the routine work in these laboratories or indicate the technic that is used when alternative methods are given in the "Standard Methods for the Examination of Water and Sewage" or the "Standard Methods for the Examination of Dairy Products."

CHAPTER 39

EXAMINATION OF WATER

Samples of water are examined at the request of the directors of the divisions of the State Department of Health, the district state health officers, the district engineers, and the local health officers. Examinations are made only when the sample is collected in a container furnished by the laboratory and a complete record of the pertinent sanitary conditions at the source of the supply accompanies it or is on file in the laboratory. Forms for recording the data are provided with each container.

Public Water Supplies.—As an aid in supervision and to provide data on which the Division of Sanitation may base recommendations for improvements, samples from all public water supplies are examined at scheduled intervals. Chemical examinations are made at least annually; bacteriologic examinations usually four, six, or twelve times a year. The frequency of sampling depends upon the results of previous examinations and upon known sanitary and operating conditions. Supplies obtained from surface sources are, in general, examined at monthly intervals. When approved county or city laboratory service is available and reports of monthly or more frequent bacteriologic examinations at these laboratories are submitted to the Department, the supplies are listed for the minimum frequency of sampling. Occasionally when specific information is desired in relation to a particular problem, the frequency of sampling is increased temporarily. Included in this routine sampling schedule are the water supplies of state institutions and of a number of selected schools, and other supplies over which close supervision is necessary in the interests of the public health.

The laboratory notifies the designated collector—the health officer or water department official—by postal card when a container is sent and when the sample should be submitted. Samples sent from public supplies under this schedule are mailed without refrigeration by parcel post, special delivery, in a container of special design; in this way delivery within twenty-four hours is assured. Experience has indicated that in water not seriously polluted bacterial changes during unrefrigerated storage for twenty-four hours are of little significance. Samples without special delivery postage are not examined and another specimen is requested.

Samples from camps and hotels are mailed in a similar manner. They are examined bacteriologically, and, as an aid in the interpretation of the results of these tests, the concentration of chloride and nitrate is determined.

Private Water Supplies.—Individuals are referred to the local health officer. If in his judgment examinations to determine the sanitary quality of the water are desirable, they are made, but only if he collects the samples in containers supplied by the laboratory, and furnishes a record of the sanitary conditions at the source of the supply. Samples from private sources are examined only for the sanitary quality of the water; for mineral analyses the owner of the supply is referred to a private laboratory for a special study of the particular problem.

Examinations are begun as soon as practicable after receipt of the sample. All samples received on holidays, Sundays, and after laboratory hours are placed in the cold room by the person receiving them. All samples for bacteriologic examination are kept in the cold room until examined. An accession number is assigned to each sample at the time the outfit is opened. This number is stamped on the container and on all identification, survey, and work forms associated with the sample. Sampling outfits and survey forms are described in "Appendix C," p. 925.

PHYSICAL AND CHEMICAL EXAMINATIONS

Physical and chemical examinations are usually made of samples of water from recently constructed public and institutional supplies and from privately owned supplies of which no recent chemical examination has been made. Samples for chemical examination are also submitted at least annually from all public water supplies in the state. In general, the procedures described in the Standard Methods for the Examination of Water and Sewage are followed (1). The chemicals used are of reagent grade and meet the specifications of the American Chemical Society. The various determinations that are made routinely are given below, with brief directions outlining the particular technic followed. Investigations of problems concerned with the operation and treatment of water supplies require many special chemical examinations, which are made in accordance with standard methods when they are available. Frequently, however, methods must be developed to meet the needs of a particular investigation.

PHYSICAL EXAMINATION

Color.—Determine by comparison with platinum cobalt standards in 50-ml. Nessler tubes. To protect the tubes from contamination by dust, cover them with 20-mm. polariscope-tube cover glasses held in place by cellulose seals (Celon). If the sample is turbid, centrifuge to remove suspended matter before making observation of the color, or filter through a clean Berkefeld filter. Express results as parts per million to the nearest whole number, interpolating between standards when necessary.

Turbidity.—Determine by comparison with silica standards prepared from a suspension of fuller's earth in distilled water and standardized in a Jackson candle turbidimeter. In determining turbidity between 5 and 25 p.p.m. compare with standards in 1-gallon bottles similar to those in which chemical samples are received. Make dilutions of samples with turbidity between 25 and 100 and compare with standards. Use a Jackson candle turbidimeter to measure turbidities greater than 100 p.p.m. Express results as parts per million to the nearest whole number, interpolating between standards when necessary.

Odor.—Odors are expressed in characteristic terms (1) and in intensities of 1 to 5.

Cold. Determine in a partially filled bottle after shaking. Record the quality and intensity of odor.

Hot. Heat the water almost to the boiling point in a partially filled tall-form 400-ml. lipless beaker or an Erlenmeyer flask covered with a watch glass. Cool to about 65°C. and determine the odor. Record the quality and intensity of odor.

CHEMICAL EXAMINATION

Ammonia Nitrogen.—Add 1 ml. of 5 M ammonia-free phosphate buffer solution, pH 7.4, to 500 ml. of the water in a distilling flask. Distill off three tubes of 50 ml. each. Nesslerize. If the third tube contains more than 0.002 mg. of ammonia, distill one or more additional 50-ml. portions. Compare the Nesslerized tubes with permanent standards (1). For the standards use 50-ml. Nessler tubes protected from dust by 20-mm. polariscope-tube cover glasses held in place by cellulose seals. Report the parts per million indicated by the sum of the readings of the tubes.

Albuminoid Nitrogen.—To the residue from the distillation of the free ammonia, add 50 ml. of alkaline potassium permanganate, connect with the condenser, and distill five portions of 50 ml. each. Nesslerize and compare with the standards. Report the parts per million indicated by the sum of the readings of the tubes after subtracting the blank reading representing the nitrogen content of the reagents used.

Nitrite Nitrogen.—Treat colored and turbid waters with aluminum hydroxide and filter before testing. Add 2 ml. of sulfanilic acid solution and 2 ml. of α-naphthylamine acetate solution to 100 ml. of the water in a short-form 100-ml. Nessler tube. Compare with standard nitrite solutions treated with the same reagents in similar jars, or permanent standards protected from dust by glass covers held in place by cellulose seals. Report to the nearest 0.001 as parts per million, interpolating between standards when necessary.

Nitrate Nitrogen.—Treat colored or turbid water with aluminum hydroxide and filter. Evaporate to dryness 50 ml. or smaller volumes, depending on the probable nitrate content, in a porcelain dish on a water bath. Moisten residue with 2 ml. of phenoldisulfonic acid. Rub with a glass rod to ensure intimate contact. Wash into a 2-ounce wide-mouthed square bottle of clear glass by adding 24 ml. of distilled water with a special pipette. Follow with 24 ml. 5 N sodium hydroxide reagent added similarly. Compare in identical bottles with permanent color standards prepared as described in Table 69. View through the side. Report to the nearest 0.01 as parts per million, interpolating between standards when necessary.

TABLE 69

Determination of nitrate nitrogen
Preparation of permanent color standards

NITRATE AS N	STANDARD COLOR SOLUTION	NITRATE AS N	CONCENTRATED COLOR SOLUTION
Micrograms	*ml.*	*Micrograms*	*ml.*
0	0 + b*	40	2.1
1	0.6 + b	50	2.6
2	1.2 + b	70	3.4
4	2.2 + b	100	4.7
6	3.3 + b	200	8.5
10	5.5 + b	300	12.0
20	10.8 + b	400	15.5
30	16.1 + b	600	22.0
		750	26.8
		1000	34.3
		1500	48.5

* b is amount to match blank for each lot of reagent; 0 to approximately 0.4 ml. The volumes of color solution are diluted to 50 ml. with dilute hydrochloric acid (1 part concentrated HCl plus 4 parts of distilled water) and dispensed in wide-mouthed 2-ounce French square bottles with molded screw caps having acid resistant liners. Make file mark at 50-ml. level. Replace with distilled water any loss due to evaporation on standing.

If the portion examined contains more than 3 mg. of chloride, nearly neutralize the alkalinity with 0.02 N sulfuric acid and treat with silver sulfate free from nitrate to remove all but 0.1 mg. of chloride. Add aluminum hydroxide, filter, and wash with a small amount of distilled water. Evaporate to dryness, moisten with 2 ml. of phenoldisulfonic acid, and proceed as above.

Preparation of Permanent Color Standards

Concentrated color solution.—Clean an ampoule containing 15 grains (0.972 grams) of gold chloride, ($AuCl_3 \cdot 3H_2O$); break in a 50-ml. volumetric flask and make to volume with distilled water.

Standard color solution.—Dilute 5 ml. of concentrated solution to 50 ml. with distilled water.

Oxygen Consumed.—In a flask place 100 ml. of the sample, or, when necessary, a smaller volume diluted with distilled water. Add 10 ml. of dilute sulfuric acid (1:3) and 10 ml. of standard potassium permanganate solution. Submerge the flask in boiling water for thirty minutes. Remove from bath and add 10 ml. of standard ammonium oxalate solution. Titrate the excess of ammonium oxalate with the permanganate solution. This amount of permanganate is equivalent to that consumed by the readily oxidizable substances in the sample. When the amount of permanganate in excess is less than one-half of the amount originally added, a smaller sample or a larger volume of standard permanganate solution should be used. Report to the nearest 0.1 as parts per million of oxygen.

Chloride.—Clarify turbid and colored samples with aluminum hydroxide. Concentrate by evaporation samples assumed to contain less than 10 p.p.m. chloride. (Evaporate 250 ml. to a volume of 50 ml. in a 6-inch white porcelain evaporating dish.) Add 1 ml. of potassium chromate indicator and titrate with standard silver nitrate solution. Each milliliter is equivalent to 0.5 mg. chloride radical. Report results as parts per million of chloride.

Total Hardness.—Determine by the soap method. Standardize alcoholic soap solution against calcium chloride made from calcite (calcium carbonate) treated with hydrochloric acid and evaporated several times to remove excess acid. Report results as parts per million of calcium carbonate.

Alkalinity.—Titrate with 0.02 *N* sulfuric acid. Use erythrosin and chloroform as an indicator for waters containing aluminum or iron sulfates. Methyl orange may be used when these salts are absent. Reports results as parts per million of calcium carbonate.

Hydrogen-Ion Concentration.—Determine colorimetrically, using indicator of proper range and compare with permanent, standard, glass, color disks. When samples are very turbid or highly colored, make the determination by electrometric methods.

Free Carbon Dioxide.—Titrate rapidly 100 ml. of the sample with 0.0227 *N* sodium hydroxide in a short-form 100-ml. Nessler tube, using phenolphthalein as an indicator. Report results as parts per million of carbon dioxide.

Iron. Thiocyanate Method (2).—

Potassium thiocyanate (KCNS)	300	grams per liter
Hydrochloric acid, concentrated	345	ml. diluted to 1 liter
Potassium permanganate ($KMnO_4$)	6.3	grams per liter

To 30 ml. of water sample in a 2-ounce French square bottle add 20 ml. hydrochloric acid. Place in a hot water bath. Hold for twenty minutes just below the boiling point. Cool and add a few drops of potassium permanganate, or just enough to maintain a pink color for one minute. Add 5 ml. of potassium thiocyanate and compare with standards in 2-ounce French square bottles. Make the final reading promptly.

When the iron content is below 0.40 p.p.m., transfer the sample to a 50-ml. Nessler tube for comparison with the set of standards in Nessler tubes for lower concentrations of iron.

Preparation of Permanent Color Standards

Cobalt chloride ($CoCl_2 \cdot H_2O$)	48 grams
Hydrochloric acid, concentrated	200 ml.
Potassium chloroplatinate (K_2PtCl_6)	4 grams
Hydrochloric acid, concentrated	200 ml.
Copper chloride ($CuCl_2 \cdot 2H_2O$)	12 grams
Hydrochloric acid, concentrated	100 ml.

Dissolve each salt in a minimal amount of distilled water, add the acid, and dilute to 1 liter. Combine the color solutions as indicated in Table 70 and dilute to 50 ml. with distilled water.

TABLE 70

Determination of iron
Preparation of permanent color standards

A. For use in 2-ounce French square bottles

EQUIVALENT CONCENTRATION OF IRON AS Fe IN 30-ML. SAMPLE	PLATINUM SOLUTION	COBALT SOLUTION	COPPER SOLUTION
p.p.m.	*ml.*	*ml.*	*ml.*
0.40	1.90	1.00	0.20
0.60	2.60	1.90	0.80
1.0	4.60	3.80	4.60
1.5	6.90	6.15	6.60
2.0	8.95	8.45	8.00
2.5	11.0	10.8	9.20
3.0	12.6	13.2	10.2
4.0	15.6	17.8	11.6

B. For use in 50-ml. Nessler tubes

EQUIVALENT CONCENTRATION OF IRON AS Fe IN 30-ML. SAMPLE	PLATINUM SOLUTION	COBALT SOLUTION	COPPER SOLUTION
p.p.m.	*ml.*	*ml.*	*ml.*
0.03	0.40	0.47	1.00
0.10	0.75	0.80	1.56
0.20	1.25	1.25	1.33
0.30	1.63	1.72	1.15
0.40	1.95	2.17	1.00
0.50	2.20	2.65	0.80

Manganese. Periodate Method.—

Sulfuric Acid Reagent

Sulfuric acid, concentrated	120 ml.
Potassium periodate (KIO_4)	2 grams

Dilute 120 ml. of concentrated sulfuric acid to about 2 liters with water double distilled in all glass apparatus in a 3-liter Erlenmeyer flask and add 2 grams of potassium periodate. When this is completely dissolved, heat to boiling. Hold in a boiling water bath for thirty minutes. Protect from dust. Dissolve 1 additional gram of potassium periodate in each 100 ml. of reagent immediately before it is used. Heat on a water bath if necessary to aid solution of the chemical.

Add from 0.75 to 1 ml. of concentrated sulfuric acid to 200 ml. of sample in a 210-ml. porcelain casserole, and evaporate on a water bath to concentrated sulfuric acid. To the residual acid solution add successive portions (from 1 to 2 ml.) of 30-per-cent hydrogen peroxide to oxidize organic matter. Repeat if necessary until sample is nearly colorless. Heat under hood until fumes of sulfuric acid are evolved. Do not permit all the acid to vaporize. Cool and dilute with 25 ml. of sulfuric acid reagent. Cover the casserole, heat to boiling and hold on a steam bath for thirty minutes. Remove and let cool. If there is a distinct pink color, transfer to clear 2-ounce French square glass-stoppered bottle. Rinse with sulfuric acid reagent and make up to 20-ml. mark. Compare with standards by viewing horizontally. If color is below lowest bottle standard, transfer to Nessler tube and compare with standards in similar tubes by viewing through length of the tube. Interpolate between readings of standards when necessary. Report as parts per million of manganese. This technic is not sensitive when used with amounts of manganese less than 0.01.

Preparation of Permanent Color Standards

Standard manganous sulfate

Potassium permanganate ($KMnO_4$)	0.1438	grams
Sulfuric acid, N	9	ml.
Sodium sulfite ($NaSO_3$)	0.5	gram

Dissolve 0.1438 grams of potassium permanganate in water double distilled in all glass apparatus. Add 9 ml. of normal sulfuric acid and then 0.5 gram of sodium sulfite. Boil off the excess sulfur dioxide and dilute to exactly 100 ml. One milliliter is equal to 0.5 mg. manganese.

Concentrated color solution

Sulfuric acid, concentrated	1.2	ml.
Sulfuric acid reagent	30	ml.
Standard manganous sulfate	20	ml.

Add 1.2 ml. of concentrated sulfuric acid and 30 ml. of sulfuric acid reagent (made as above) to exactly 20 ml. of standard manganous sulfate. Heat to boiling and hold for thirty minutes in a boiling water bath. Cool and dilute to 100 ml. with sulfuric acid reagent. This solution contains 0.1 mg. manganese per milliliter. If protected from dust and light and stored in a cool place, it will remain unchanged for six months or longer.

Standard color solution

Dilute 1 ml. of concentrated solution to 100 ml. with sulfuric acid reagent. This solution contains 0.001 mg. manganese per milliliter. Prepare standards as indicated in Table 71.

TABLE 71

Determination of manganese
Preparation of permanent color standards

EQUIVALENT CONCENTRATION OF MANGANESE AS Mn IN 200-ML. SAMPLE	CONCENTRATED COLOR SOLUTION	STANDARD COLOR SOLUTION
p.p.m.	*ml.*	*ml.*
0.010*		2.0
0.015*		3.0
0.030†		6.0
0.05‡		10.0
0.10		20.0
0.15	0.3	
0.30	0.6	
0.50	1.0	
1.00	2.0	
1.50	3.0	
3.00	6.0	
5.00	10.0	
10.0	20.0	

* Comparison made in 50-ml. Nessler tubes.

† Comparison made in 50-ml. Nessler tubes or 2-ounce French square glass-stoppered bottles.

‡ From this point comparison made in 2-ounce French square bottles only.

Dilute all standards to 20 ml. with sulfuric acid reagent. Make up loss from evaporation with water double-distilled in all glass apparatus.

Residual Chlorine

Measure total residual chlorine by the ortho-tolidine test (1). Use the ortho-tolidine-arsenite test when differentiation between free available and combined chlorine is required and when oxidized manganese or iron causes a false color with the standard ortho-tolidine test (3, 4).

Prepare the ortho-tolidine and sodium arsenite reagents and permanent color standards in accordance with the procedure given in Standard Methods for the Examination of Water and Sewage.

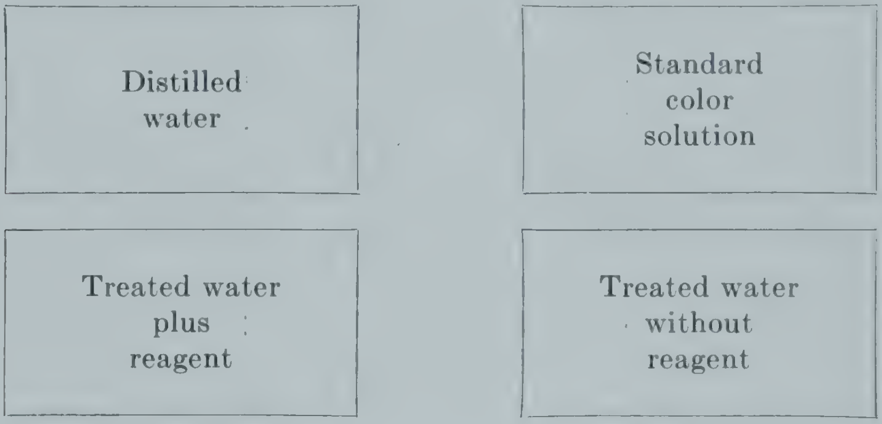
Distilled water of no chlorine demand.—Measure 4 liters of distilled water, 10 ml. of 25-per-cent sulfuric acid, and 10 ml. of 0.125 *N* potassium permanganate into a 5-liter boiling flask with standard taper joint. Insert a glass condenser and distill into a 3-liter Erlenmeyer flask until nearly full. Add 15 ml. of a 100-p.p.m. solution of chlorine in distilled water and let stand overnight. Boil down to about 2 liters and let cool. Test for nitrite, ammonia, chlorine, and chlorine demand. No amounts measurable by the standard tests should be found, nor should there be an odor resembling that of ozone or oxides of nitrogen.

Ortho-tolidine (OT) Test.—Collect in 2-ounce French square bottles two 50-ml. portions of a sample of the chlorinated water. Add one dropperful (0.75 ml.) of the ortho-tolidine reagent to one portion and mix thoroughly.

FIG. 59. RESIDUAL CHLORINE BY DOUBLE-BOTTLE TECHNIC

Light source

(North light or light from a fluorescent daylight lamp diffused by transmission through opal glass or by reflection from a dull surface)



Observer

Each block represents a 2-ounce French square bottle containing 50 ml.

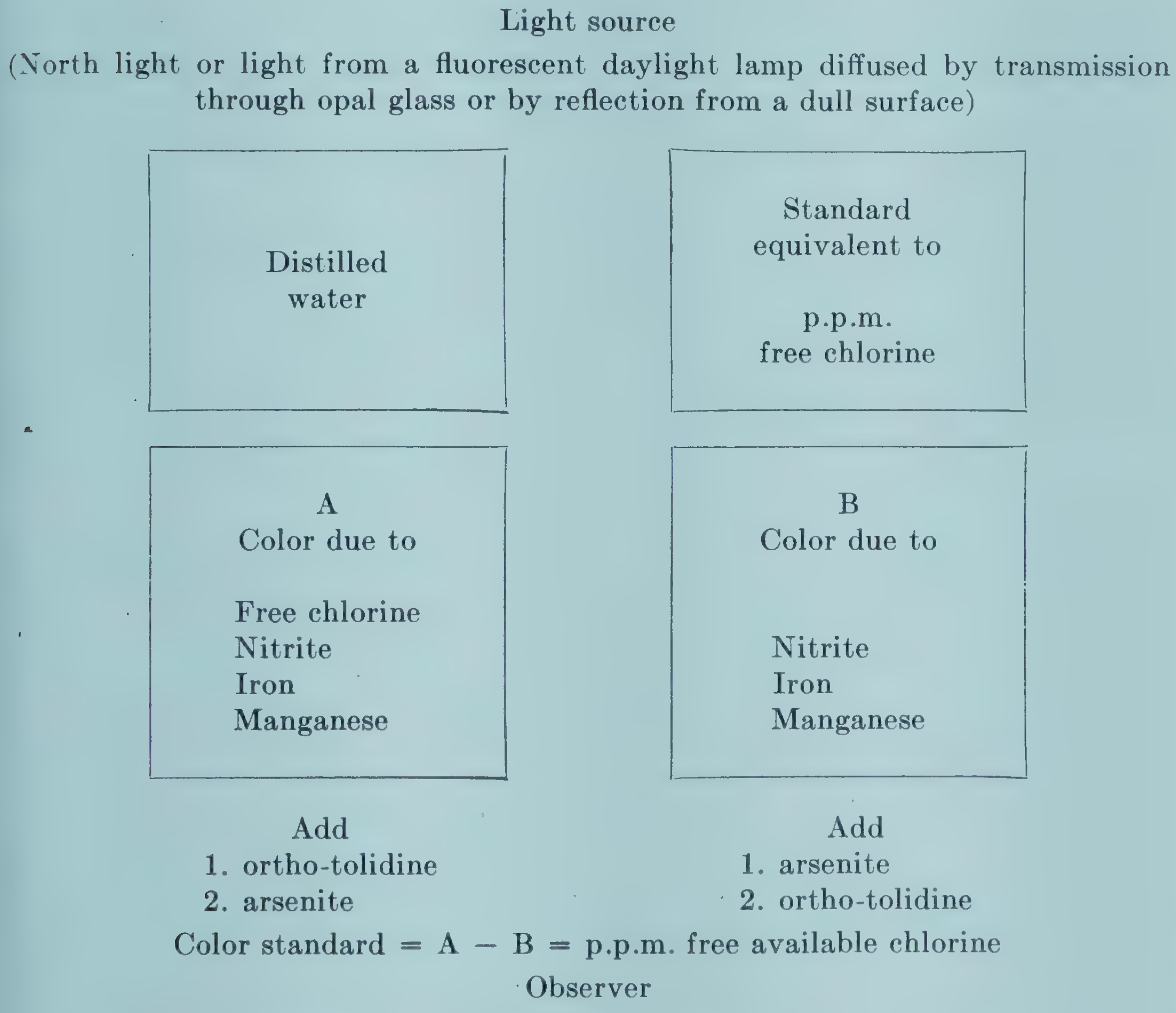
Allow the sample to stand five minutes in the dark. During the five-minute period, warm if necessary to 20°C. in a bath of slightly warmer water or by holding in the hand. Compare the resulting color with the standard solutions and record the results of the test as parts per million residual chlorine, interpolating between standards when necessary.

If the sample is colored or turbid, place the second portion to which reagent has not been added in front of the standard and another of distilled water in back of the sample containing ortho-tolidine before making the color comparison (see figure 59). Where color or turbidity is in excess of 100 p.p.m., the precision of the test is markedly lowered.

Ortho-tolidine-arsenite (OTA) Test.—Mark three 2-ounce French square bottles A, B, and OT. Measure 50 ml. of sample into each. Add a dropperful (0.75 ml.) of ortho-tolidine reagent to bottle marked OT. Mix by one quick shake after each addition. Add a dropperful of ortho-tolidine reagent to bottle marked A. Within fifteen seconds add a dropperful of arsenite reagent to A. Add a dropperful of arsenite to B followed by a dropperful of ortho-tolidine reagent.

If the sample is cold, warm portion OT to 20°C. in a bath of slightly warmer water or by holding in the hand. Portions A and B do not require warming.

FIG. 60. FREE AVAILABLE CHLORINE


Light source
(North light or light from a fluorescent daylight lamp diffused by transmission through opal glass or by reflection from a dull surface)
Distilled water
Standard equivalent to
p.p.m.
free chlorine
A
Color due to
Free chlorine
Nitrite
Iron
Manganese
B
Color due to
Nitrite
Iron
Manganese
Add
1. ortho-tolidine
2. arsenite
Add
1. arsenite
2. ortho-tolidine
Color standard = A − B = p.p.m. free available chlorine
Observer

Each block represents a 2-ounce French square bottle containing 50 ml.

Free available chlorine. Read one to five minutes after addition of reagent, using portions A and B (see figure 60).

False residual. The B reading alone represents the false color caused by oxidized manganese, iron, or nitrite. If the sample is colored or turbid, compare with another bottle of sample to which no reagent has been added (see figure 59).

Standard ortho-tolidine test. Read the OT portion in the usual manner five minutes after addition of reagent.

Combined chlorine. Read at five minutes, using portions OT and A (see figure 61).

FIG. 61. COMBINED CHLORINE

Light source

(North light or light from a fluorescent daylight lamp diffused by transmission through opal glass or by reflection from a dull surface)

Distilled water	Standard equivalent to p.p.m. combined chlorine
OT Color due to Combined chlorine Free chlorine Nitrite Iron Manganese	A Color due to Free chlorine Nitrite Iron Manganese

Color standard = OT − A = p.p.m. combined chlorine

Observer

Each block represents a 2-ounce French square bottle containing 50 ml.

Chlorine Demand

Measure the chlorine demand of water by determining the amount of chlorine absorbed in producing a given residual after a contact period of ten minutes.

Sodium hypochlorite solution, 1000 p.p.m. chlorine.—Dilute a commercial hypochlorite solution with water of no chlorine demand. Standardize by titration with potassium iodide and sodium thiosulfate and adjust so that the available chlorine concentration is between 950 and 1050 p.p.m. chlorine.

Sodium hypochlorite solution, 100 p.p.m. chlorine.—Dilute the 1000 p.p.m. chlorine solution 1:10 with water of no chlorine demand. Standardize as above and adjust so that the available chlorine concentration is between 95 and 105 p.p.m. chlorine. Each 0.1 ml. equals approximately 0.2 p.p.m. chlorine dose in 50 ml. of sample. Store in the cold room.

Measure four 50-ml. portions of sample into 2-ounce French square bottles. Add 0.1-, 0.2-, 0.4-, and 0.8-ml. portions of 100 p.p.m. hypochlorite solution, equivalent to 0.2, 0.4, 0.8, and 1.6 p.p.m. chlorine, mix, let stand ten minutes. Test for total residual chlorine. Repeat the test with more or less hypochlorite if necessary to obtain one residual chlorine value between 0.1 and 0.2 p.p.m. and another between 0.2 and 0.3 p.p.m. A single value may be reported if the residual is exactly 0.2 p.p.m.

Report the chlorine dose, demand, and residual as parts per million of chlorine.

MICROSCOPIC EXAMINATION

Microscopic examinations of water are made on unpreserved samples submitted by the staff of the Division of Sanitation, or by the operators of public water supply systems. They are also made on samples taken in studies on the pollution of streams and the operation of sewage treatment plants. Samples from public water supplies and streams are submitted in 1-gallon bottles and also serve as samples for chemical examinations. Deposits in streams or filters and growths in pipes or sewage plant units are collected in the 4-ounce bacteriologic sample bottles or in preserve jars. Examination is made as soon as possible after receipt of the sample, using the Sedgwick-Rafter method (1).

Apparatus.—Sedgwick-Rafter filtering funnel. Silk bolting cloth disks. Berkshire filtering sand of such a size that it will pass a screen having 60 meshes per linear inch and be retained on a screen having 120 meshes per linear inch. Counting cell, 1 ml. capacity, 50 by 20 mm. by 1 mm. in depth, inside dimensions. Ocular micrometer.

The microscope must be fitted with an ocular micrometer. Use a stage micrometer to determine the combination of objective, ocular, and tube length required for the area on the slide covered by the ocular micrometer to be exactly 1 sq. mm. The smallest square on the ocular micrometer will then cover an area of 20 by 20 microns or 400 square microns. This is the Areal Standard Unit.

Concentrate samples from public water supplies and streams. Make a quantitative count on public water supply samples.

Concentration.—Assemble Sedgwick-Rafter funnel. Add filter sand to depth of one-half inch. Filter 500 ml. of carefully mixed sample. Use a smaller volume when a heavy concentration of organisms is indicated. Transfer sand to a beaker and wash several times; decant after each washing and make up to a final volume of 10 ml.

Samples from public water supplies.—Mix concentrate by rotation. Transfer 1 ml. to the counting cell. Examine under a microscope which has been standardized as above. Count 10 fields selected at random and classify the organisms.

Determine the Areal Standard Units occupied by amorphous matter in the 10 fields counted. Express the results in Areal Standard Units per milliliter for each organism identified and for amorphous matter.

Samples from streams.—Concentrate 500 ml. or less of sample. Place 1 ml. of concentrate in counting cell. Make a qualitative examination only; classify and tabulate the organisms, indicating the predominant ones, but do not make a count.

Deposits in streams and filters; growths in pipes and sewage plant units.—Examine directly without concentration several 1-ml. portions. Small amounts of filamentous growths may be placed on a microscope slide and teased apart to permit more adequate examination of the growth.

Add a few drops of dilute hydrochloric acid to dissolve the iron from forms encrusted with iron. Examine under high power lens if necessary to reveal the structure. Classify and tabulate the organisms, indicating the predominant ones, but do not make a count.

For assistance in the identification of the organisms found, consult standard reference texts (5–10).

BACTERIOLOGIC EXAMINATION

Samples of water for bacteriologic examination are collected and shipped to the laboratory as already described. Sterile bottles distributed for the collection of samples from swimming pools or bathing areas and from all public and institutional water supplies contain 0.1 ml. of a 1-per-cent solution of sodium thiosulfate. Such bottles have a paper cover-cap. Other sterile bottles without thiosulfate are capped with metal foil. After receipt samples are kept under refrigeration until examined. Outfits are opened one at a time to avoid the possibility of interchange. Shake the sample bottles vigorously twenty-five times before removing portions for examination.

Tests for Bacteria of the Coliform Group

Follow in general the procedures for the completed test as given in Standard Methods for the Examination of Water and Sewage (1). Inoculate beef-extract lactose broth in Dunham fermentation tubes with the following volumes as indicated: *F14C* for the 10-ml. portion and *F14B* for smaller portions.

Potable supplies: 5 tubes 10 ml.; 1, 1 ml.; 1, 0.1 ml.

Swimming pools: 3 tubes each 10 ml., 1 ml., 0.1 ml., 0.01 ml.

Bathing beaches: 3 tubes each 1 ml., 0.1 ml., 0.01 ml., 0.001 ml., and 0.0001 ml.

Grossly polluted water, and polluted streams: three portions of each volume selected, choosing a range such that the coliform group will be absent in the smallest volume used and present in the largest.

Incubate all tubes for forty-eight hours at 35°–37°C. Record gas production for each tube at the end of eighteen to twenty-four hours and again at the end of forty-eight hours' incubation. Make subcultures from the primary fermentation tubes showing gas in any amount after eighteen to twenty-four hours' incubation or in amounts of at least 10 per cent of the volume of the inner tube after forty-eight hours' incubation. Make subcultures in fermentation tubes containing peptone bile medium with brilliant green and lactose (*F24*). If five primary fermentation tubes are inoculated, make subcultures from two showing the requisite amount of gas; and from one tube if three is the total number inoculated. However, if gas in an amount occupying at least 10 per cent of the volume of the inner tube is not produced in one or more of the subcultures within eighteen to twenty-four hours, prepare other subcultures from different primary fermentation tubes containing the same volume of sample and producing the requisite amount of gas. Record the amounts of gas produced in the subcultures at the end of eighteen to twenty-four hours' incubation and again at the end of forty-eight hours' incubation.

Presumptive test.—Consider gas production in an amount occupying 10 per cent or more of the volume of the inner tube of the primary fermentation tube within forty-eight hours' incubation as "presumptive" evidence of the presence of bacteria of the coliform group in the original sample.

Confirmed test.—Consider gas production in an amount occupying 10 per cent or more of the volume of the inner tube of the brilliant green bile medium subculture within forty-eight hours as "confirmed" evidence of the presence of bacteria of the coliform group in the original sample.

Consider all primary fermentation tubes containing a given volume of sample and producing gas in an amount occupying at least 10 per cent of the volume of the inner tube as "confirmed" if any one of these tubes is confirmed.

Completed test.—Carry out the "completed" test on any samples concerned with special investigations of water supplies where complete data concerning the degree or type of pollution is required. Dilute in 10 ml. of sterile tap water a loopful of culture from a confirmatory tube that shows gas in an amount occupying at least 10 per cent of the volume of the inner tube. With a glass rod, streak a loopful of this diluted culture over the surface of an Endo (*F52B*) or eosin methylene blue agar plate (*F53*). Incubate the plates for eighteen to twenty-four hours at 35°–37°C. If typical or atypical coliform colonies develop, select one or more and prepare two subcultures from each—one on a slant of beef-extract agar with Andrade's indicator and lactose (*F50A*) and the other in a beef-extract lactose broth (*F14B*) fermentation tube. If there is no growth on the plate or no typical or atypical coliform colonies develop within twenty-four hours, reincubate the plate for twenty-four hours. If typical or atypical colonies then appear, select one or more for subculturing. If there is no growth within forty-eight hours, or the growth is not coliform, discard the plate.

Incubate the agar slant and the fermentation tube cultures for forty-eight hours. If gas is produced within this period in the confirmatory fermentation tube containing beef-extract lactose broth in an amount occupying at least 10 per cent of the volume of the inner tube, make a Gram-stain preparation (p. 35) from the culture on the agar slant inoculated from the same colony. Examine for the presence of Gram-negative nonsporeforming rods.

Consider gas production in the confirmatory fermentation tube in an amount occupying at least 10 per cent of the volume of the inner tube coupled with the presence of Gram-negative nonsporeforming rods in the agar slant culture as "completed" evidence of the presence of bacteria of the coliform group in the original sample.

Consider lack of gas production or gas production of less than the prescribed amount in the confirmatory fermentation tube or the presence of other than Gram-negative nonsporeforming rods in the agar slant culture as evidence of the absence of bacteria of the coliform group in the original sample.

Results of Tests.—Report the density of the bacteria of the coliform group in the original sample as the most probable number (M.P.N.) per 100 ml.; compute the most probable number according to the tables of Hoskins (1, 11).

Standard Plate Count

Plate 1 ml. of the sample in beef-extract tryptone glucose agar (*F49C*). If it is anticipated that the water may contain three hundred or more bacteria per milliliter, dilute the sample to 1:10, 1:100, or more if necessary, with sterile tap water; plate a series of dilutions calculated to yield at least one plate within the proper range. In the routine examination of potable waters, plate 1 ml. of the sample and 1 ml. of a 1:10 dilution.

Incubate the agar plates for twenty-four hours at 35°–37°C.

Count the plates that contain between 30 and 300 colonies. If it is necessary to count a plate with a greater number of colonies, select a representative fractional area bearing 150 to 200 colonies and multiply the count by the proper factor to obtain the number of colonies on the plate. If the plate with the largest volume inoculated contains less than 30 colonies, record the count as found.

Take the plate count multiplied by the factor of dilution as the number of bacteria per milliliter in the water. Make all counts in an illuminated counting device with a lens of 1.5 magnifying power.

Results of the Test.—Report the count as the number of bacteria per milliliter growing on agar within twenty-four hours at 35°–37°C. Use only two significant digits in reporting the result.

REPORTING RESULTS

The results of each routine test are typed under the laboratory serial numbers on printed forms that provide also for recording the specific location from which the sample was taken, the source of the sample, name of the collector, and the dates of collection and receipt. Reports on samples collected from public water supplies by engineers of the Department or by local officials are sent without comment to the director of the Division of Sanitation who in turn sends them to the municipal officials, interpreting the findings in the light of the sanitary survey that accompanied the sample or that is on file in his office.

Samples from water supplies of schools, camps, summer hotels, institutions, and from privately owned sources, when submitted by the district engineer or other member of the staff of the Division of Sanitation, are reported in the same manner to the director of that division. Two extra copies of the report of an analysis of a sample from a school supply are sent to the director of the Division of Sanitation for transmittal to the State Department of Education. A copy of the report of examination of a sample collected in connection with illness is sent to the Division of Communicable Diseases.

Reports of examinations of samples collected by health officers from private supplies are sent directly to them with an interpretation of the results. Reports of examinations of samples collected by health officers from all other sources are submitted to them without interpretation through the Division of Sanitation.

EXAMINATION OF ICE

The bacteriologic examination of ice is rarely undertaken since a sanitary survey and laboratory examination of the source of water from which the ice is produced yield more significant information concerning its potential sanitary quality than the direct bacteriologic examination.

A representative piece of ice weighing about 100 pounds (more, if shipped from a distance in warm weather), must be sent to the laboratory packed in sawdust.

If it is natural ice, the sample must be accompanied by a record of the sanitary survey of the source of the water from which it was cut, giving detailed information concerning any sources of pollution located near the ice field, or upstream from it.

If the sample is of artificial ice it must be accompanied by adequate identifying data and information concerning its source and the treatment of water from which it was produced.

BACTERIOLOGIC EXAMINATION

Remove the sawdust from the cake of ice and rinse the surface with sterile water. Remove the outside surfaces by chipping with an axe that has been sterilized by flaming, and split the ice at right angles to the original horizontal plane. Remove chips from this newly fractured surface and place them in a 6-ounce, sterile, wide-mouthed bottle, using a sterile metal spoon.

If the cake contains snow ice and clear ice in distinct layers, a sample of each may be chipped from the cake and placed in separate bottles.

Allow the ice in the bottles to melt completely at room temperature. Proceed with the tests and report the results as in the examination of a sample of potable water.

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CHAPTER 40

EXAMINATION OF SEWAGE, SEWAGE SLUDGE, INDUSTRIAL WASTES, AND POLLUTED WATER

SEWAGE, INDUSTRIAL WASTES, AND POLLUTED WATER

Samples of sewage, industrial wastes, and polluted water are examined only when submitted by engineers or other members of the staff in investigations of the operation of sewage treatment plants or of stream pollution. They must be accompanied by the necessary identifying and descriptive data regarding the source, type of sewage treatment, and method of operation at the time of sampling. Reports are made to the Division of Sanitation. The procedures given in Standard Methods for the Examination of Water and Sewage (1) are followed. The determinations made routinely are given below with brief directions to indicate the particular technic found most satisfactory in this laboratory. Investigations of special problems concerned with sewage treatment frequently require additional chemical tests which are also made in accordance with the methods advocated by the Association whenever they are available.

CHEMICAL EXAMINATION

Sewage and Industrial Wastes.—Only representative samples are examined. They should be composites of specimens taken at intervals of one hour or less, over a period determined by the nature of the investigation; generally twelve or twenty-four hours. When possible they should be integrated according to the rate of flow. Samples should be collected in duplicate in glass-stoppered bottles (1-gallon capacity) furnished by the laboratory. Observation of the physical appearance and determination of dissolved oxygen, pH value, residual chlorine, temperature and the initial steps of the biochemical oxygen demand procedure should be made at the time of collection. One of the duplicate portions should be preserved by the addition of 1 ml. of concentrated sulfuric acid (specific gravity 1.84) per liter of sample and the second portion by 5 ml. of chloroform per liter. Samples should be refrigerated from the time of collection and transported to the laboratory as soon as possible.

Use the sample preserved with sulfuric acid to determine oxygen consumed, grease, chloride, and the compounds of nitrogen. Use the portion preserved with chloroform for the determination of solids, alkalinity, and turbidity.

Polluted Water.—Samples from streams should be collected at points selected with regard for added pollution, hydrologic, and physical features.

They should be collected at mid-depth in the main channel, except that in large rivers samples from various depths and distances from shore are necessary to obtain a representative cross section. All samples should be taken in glass-stoppered bottles (1-gallon capacity) furnished by the laboratory. Determinations of dissolved oxygen, carbon dioxide, pH value, temperature, and the initial steps of the biochemical oxygen demand procedure should be made at the time of sampling. Samples should be transported to the laboratory as quickly as possible and should be accompanied by pertinent data obtained from a complete sanitary survey of the stream and its environs.

Samples Examined at Time of Collection

Dissolved Oxygen.—Dissolve one gram of sodium azide in 100 ml. of alkaline potassium iodide solution and follow the standard Winkler procedure. Report to the nearest 0.1 as parts per million of oxygen.

Free Carbon Dioxide.—Titrate 100 ml. sample in a short-form Nessler tube with 0.0227 *N* sodium hydroxide, using phenolphthalein as indicator. Report to the nearest 0.1 as parts per million of carbon dioxide.

pH Value.—Determine colorimetrically; compare with permanent, standard, glass, color disks in a comparator.

Residual Chlorine.—Use ortho-tolidine reagent (15-per-cent acid) and compare with permanent, standard, glass, color disks in a comparator.

Biochemical Oxygen Demand.—Prepare a series of dilutions by adding measured volumes of sewage to bottles of 300-ml. capacity and filling with dilution water. Estimate quantities so that at least one dilution will provide a 40- to 70-per-cent reduction of the dissolved oxygen content after five days' incubation at 20°C. For dilution water use aerated and stored distilled water buffered to pH 7.2 and containing ammonium sulfate. If dilutions greater than 1:100 are necessary prepare a dilution of the wastes and use measured portions of this dilution to prepare the series. Incubate with the samples a control containing dilution water only. Use the dissolved oxygen of this control as the initial concentration of dilutions. If dilutions of less than 10 per cent are used, determine the dissolved oxygen concentration of the sample and calculate the initial dissolved oxygen of the dilution. If the original sample is known to be sterile, neutralize or remove bactericidal agents before preparing dilutions, and seed the dilution water with sewage organisms by the addition of 5 ml. of settled sewage to each four liters.

Apparatus and reagents are carried in a field kit designed for the purpose. The box is of $\frac{1}{4}$-inch white wood and is 22 inches long, $8\frac{1}{2}$ inches wide, and $8\frac{1}{2}$ inches high. (See Fig. 62.) The top and front are hinged to open outward and a step $2\frac{1}{2}$ inches high and 4 inches wide is built into the bottom at the rear. Reagent bottles are fitted

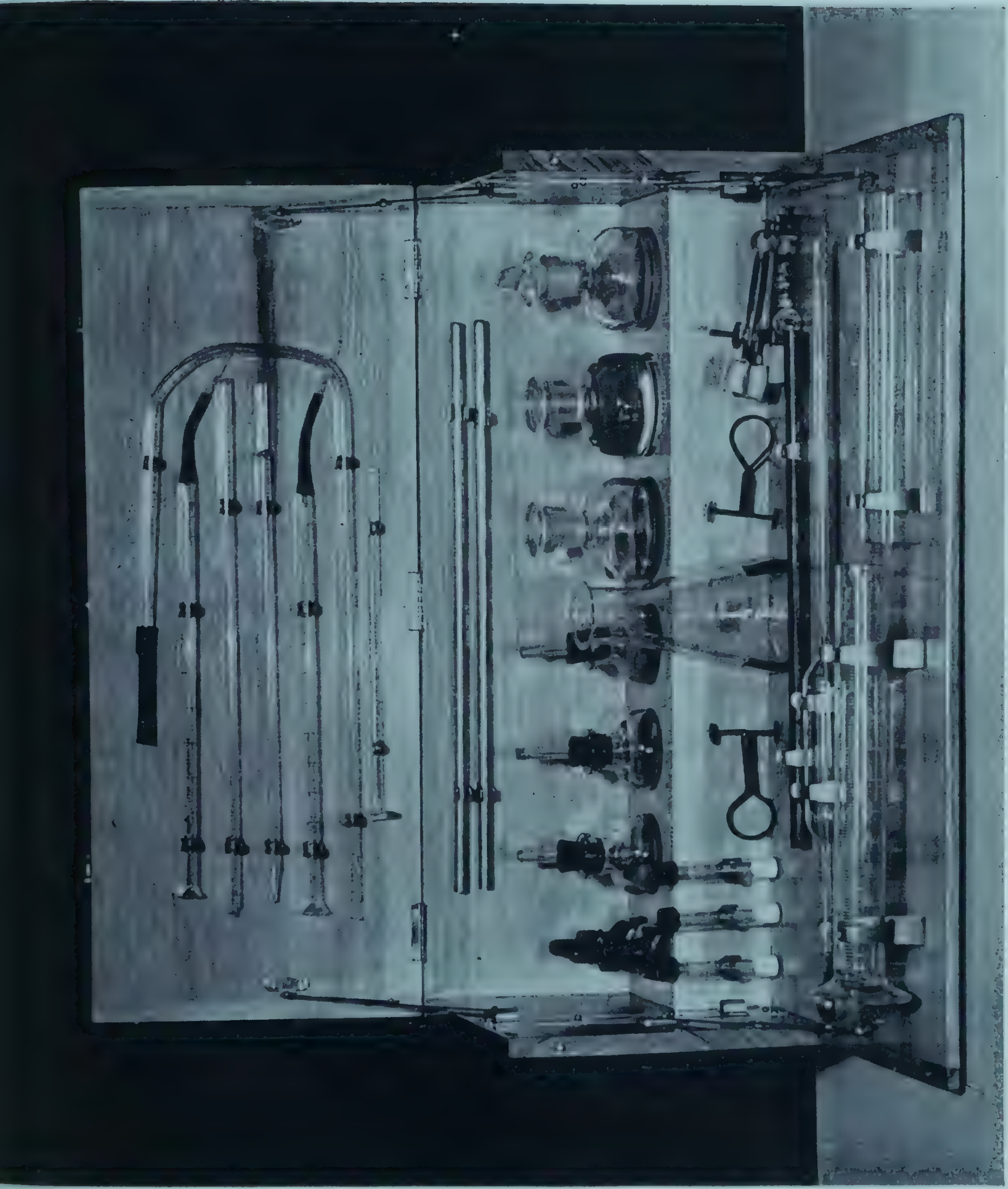

FIG. 62. LIGHT-WEIGHT FIELD KIT FOR STUDIES OF STREAM POLLUTION

into holes of the proper size cut into this step. Attached by clips to the bottom of the case in front of the step are a 250-ml. Erlenmeyer flask and an armored thermometer. Burette holders and pinchcocks are carried loose in the bottom. Fastened to the rear panel by clips are two brass rods which can be inserted into brass clips on the end pieces to serve as burette stands. On the front panel are attached a 100-ml. graduate, a 100-ml. short-form Nessler tube for carbon dioxide titrations, and a 50-ml. transfer pipette. The top panel carries two 10-ml. burettes, a Mohr pipette, glass tubing, and a plunger-type stirring rod. When closed the kit (14 lbs.) is carried by a web belt strung through two clips attached to each end of the box.

Samples Preserved with Sulfuric Acid

Ammonia Nitrogen.—Clarify 100 ml. of the sample by the addition of 1 ml. of zinc sulfate (0.6 *M*) and about 0.5 ml. of sodium hydroxide (6 *N*) to produce a rapidly settling precipitate. Centrifuge and Nesslerize an aliquot portion using 2.5 ml. Rochelle salt solution per tube to prevent precipitation of calcium and magnesium compounds. Compare with permanent standards, interpolating between values when necessary, and report to the nearest 0.01 as parts per million of nitrogen.

Organic Nitrogen.—Determine total nitrogen by the Kjeldahl method. Distill into 5 ml. of boric acid (saturated solution) diluted to 25 ml. until about 200 ml. of distillate has been obtained. Add 3 drops of methyl red and titrate the ammonia with 0.05 *N* sulfuric acid. A blank should be run on the reagents used and corrections made if necessary. The organic nitrogen is equal to the difference between the nitrogen found after digestion and the ammonia nitrogen. Report to the nearest 0.01 as parts per million of nitrogen.

Nitrite Nitrogen.—To 250 ml. of neutralized sample add about 1 gram of activated carbon and 3 ml. of aluminum hydroxide paste, mix, and filter. Discard the first portion of the filtrate. Use an aliquot portion of the clarified sample and proceed as in the examination of water. Report to the nearest 0.01 as parts per million.

Nitrate Nitrogen.—Use an aliquot portion of the sample clarified for nitrite nitrogen and proceed as in the examination of water. After treatment with alkali, add activated carbon and filter to remove any brown, off-shade color. Compare with permanent standards, interpolating between values when necessary, and report to the nearest 0.01 as parts per million.

Chloride.—Use an aliquot portion of the sample clarified for nitrite nitrogen and proceed as in the examination of water. If the sample contains sulfides, oxidize by heating with hydrogen peroxide in alkaline solution (pH 8.2 or greater). Cool, neutralize, dilute to original volume, and proceed with titration as in examination of water. Report to the nearest 0.1 as parts per million of chloride.

Oxygen Consumed.—Proceed as in the examination of water. To avoid the use of very small samples, prepare potassium permanganate and ammonium oxalate solutions ten times stronger than standard, and use 20-ml. volumes of reagents. Adjust the quantity of sample so that at least 25 per cent but not more than 50 per cent of the potassium permanganate reagent is reduced during digestion. Report to the nearest 0.1 as parts per million of oxygen.

Grease.—The procedure used is a modification of that proposed by Gehm (2). Warm to room temperature and neutralize 1 liter of sample to pH 7.2 to 7.4 with 18 N sodium hydroxide. Add 1 ml. of an alum solution (0.5 M) ($Al_2(SO_4)_3 \cdot 18H_2O$). Stir gently for five minutes and allow to stand until the floc settles completely, preferably overnight in the refrigerator. Fit a Büchner funnel of 11.5-cm. diameter with a layer of muslin overlaid with a No. 40 Whatman filter paper. Suspend 1 gram of diatomaceous earth in 100 ml. of distilled water and pour in the funnel. Apply vacuum and wash the mat with 1 liter of distilled water. Filter the sample through the mat, taking care to decant the clear supernatant through first. Wipe the beaker that contained the sample with a small piece of filter paper saturated with petroleum ether. Remove the mat from the funnel and wipe the funnel with a piece of filter paper moistened with petroleum ether. Remove the filter cloth from the mat. Place on a watch glass the mat containing the residue and the two pieces of filter paper used to wipe the beaker and funnel and moisten with 10 ml. of hydrochloric acid (10 per cent by volume). Dry contents of the watch glass at 103°C. in an oven and transfer to a paper extraction shell. Close the shell with glass beads and extract with petroleum ether (B.P. 30°–60°C.) in a Soxhlet extractor for twelve hours. Remove all but 1 ml. of the solvent from the flask by distillation on a water bath. Connect the flask to a source of vacuum and place it on a steam bath for fifteen minutes. Remove, cool in a desiccator, and weigh. Report to the nearest 0.1 as parts per million.

Samples Preserved with Chloroform

Total Residue on Evaporation.—Evaporate 100 ml. of sample in an ignited and weighed porcelain or platinum evaporating dish, dry to constant weight at 103°C., and weigh. Report increase in weight to the nearest 0.1 as parts per million of total residue on evaporation.

Total Volatile Solids.—Ignite the total residue on evaporation in an electric muffle furnace at dull red heat (600°C.) until organic matter is entirely volatilized. Cool in desiccator and weigh. Report the loss in weight to the nearest 0.1 as parts per million of total volatile solids.

Total Fixed Solids.—The weight of the residue after the determination of volatile solids is that of the total fixed solids. Report to the nearest 0.1 as parts per million.

Suspended Solids.—In a Gooch crucible prepare a mat about 4 mm. thick with acid-washed asbestos that has been shredded and thoroughly ignited. Dry the crucible and then ignite at 600°C., cool, and weigh. Filter 500 ml. of sample, or an aliquot portion, through the crucible (residue should not exceed 30 mg.), dry at 103°C. for one hour, cool in a desiccator, and weigh. Report increase in weight to the nearest 0.1 as parts per million of suspended solids.

Volatile Suspended Solids.—Ignite the crucible containing the suspended solids in an electric muffle furnace at 600°C. until organic matter is entirely volatilized. Cool in desiccator and weigh. Report loss in weight to the nearest 0.1 as parts per million of volatile solids.

Suspended Fixed Solids.—The weight of residue after determination of volatile suspended solids is that of the suspended fixed solids. Report to the nearest 0.1 as parts per million.

Dissolved Solids.—Report the differences between suspended solids and total solids as concentrations of total, volatile, and fixed dissolved solids.

Alkalinity.—Proceed as in examination of water. If end point with this titration is obscure, add to 100-ml. portion of sample, 5 grams of potassium acid phthalate to produce pH of 4.0 and 10 drops of methyl orange indicator. To a second 100-ml. portion, add 10 drops of methyl orange indicator and titrate with 0.02 *N* sulfuric acid (*N* sulfuric acid if alkalinity is very high) until color matches that of buffered sample diluted with volume of distilled water equivalent to amount of acid solution used in titration. Report to the nearest 0.1 as parts per million of calcium carbonate.

If the sample is colored or contains a high concentration of alkaline salts, an electrometric titration should be made. Use an electrometric pH meter with titration flask assembly. Use a measured volume of sample sufficient to cover both electrode bulb and junction tip. Add 0.02 *N* sulfuric acid (*N* sulfuric acid if alkalinity is high) from a burette in increments (0.2 to 0.5 ml.) and measure pH after each addition until the end point is definitely passed. Plot the resulting data and take as the end point either the inflection point of the curve when measuring strongly alkaline solutions or pH 4.0 when measuring alkalinity.

BACTERIOLOGIC EXAMINATION

Samples collected at appropriate intervals should be used for the bacteriologic examination of sewage. Samples of chlorinated sewage effluents should be collected in bottles to which 1 ml. of a 1-per-cent solution of sodium thiosulfate has been added prior to sterilization in order to neutralize the residual chlorine in the sample. Samples for bacteriologic examination must be carefully refrigerated and transported to the laboratory as soon as possible. Shake sample bottles vigorously twenty-five times before removing portions for examination.

Tests for Bacteria of the Coliform Group

Perform tests as in the examination of water, p. 588. Prepare a series of volumes of the sample, decimal multiples of 1 ml. Inoculate primary fermentation tubes of beef-extract lactose broth (*F14B*) with three portions of each volume. Select a range of volumes that will probably yield negative results in the smallest volume selected and positive results in the largest.

Standard Plate Count

Carry out the test as in the examination of water, p. 590. Prepare plates with beef-extract tryptone glucose agar (*F49C*). If the count is expected to be less than 300 colonies per ml., plate 1 ml. If the count is anticipated to be greater than 300 or nothing is known of its probable magnitude, plate a series of dilutions of the sample in sterile tap water of sufficient range that a count between 30 and 300 is assured.

SEWAGE SLUDGE

CHEMICAL EXAMINATION

Samples of sewage sludge are examined only when submitted by the staff of the Department in their investigations of the operation of sewage treatment plants. Samples should be submitted in wide-mouthed glass-stoppered bottles or preserve jars and must be accompanied by complete identifying and descriptive data regarding the source and the type of treatment of both sewage and sludge at the time of sampling.

The procedures described in Standard Methods for the Examination of Water and Sewage are followed. The determinations used in the routine examination of sludge are listed below with brief directions that outline the specific technic found most satisfactory in this laboratory. Frequently additional chemical tests are required in special investigations of sewage treatment, and these are also made in accordance with standard procedures whenever such methods are available.

Samples.—Care must be taken in sampling sludge to ensure the collection of representative samples. They should be refrigerated during storage.

Moisture.—To a weighed nickel or porcelain dish, add a volume of sludge (usually 50 ml.), weigh, dry to constant weight at 103°C., cool in desiccator, and weigh. The loss in weight measures the moisture. Compute to the nearest 0.1 as per cent of total sample.

Total Solids.—The weight of the solids after removal of moisture is the weight of the total solids. Compute to the nearest 0.1 as per cent of total sample.

Volatile Solids.—Ignite the total solids in an electric muffle furnace at dull red heat (600°C.) until organic matter is completely volatilized, cool, and weigh. Compute volatile solids to the nearest 0.1 as per cent of the total solids.

Mineral Residue.—Residue after determination of volatile solids. Compute to the nearest 0.1 as per cent of the total solids.

Grease.—Use the method proposed by Gehm and Trubnick (3). After determining the solids content, take a weighed portion of the wet sample equal to 1 gram of fresh solids, 3 grams of activated sludge, or 0.5 gram of scum. Add 10 ml. of hydrochloric acid (10 per cent by volume) followed by diatomaceous earth. For fresh solids use a quantity of earth equal to the weight of the dry solids, for activated sludge use one-half the weight, and for scum use twice the weight. Mix and evaporate to dryness. Scrape the dried material into a paper extraction shell. Extract with petroleum ether (B.P. 30°–60°C.) for twelve hours in a Soxhlet apparatus. Report grease as per cent of dry solids.

pH Value.—Determine electrometrically.

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(2) Gehm, H. W. The determination of grease in sewage, sludge and scum. 2. Determination of grease in sewage. Sewage Works J., 1941, **13,** 927–935.

(3) Gehm, H. W., and Trubnick, E. H. Grease in sewage, sludge and scum. 1. Determination of total and non-saponifiable grease. Sewage Works J., 1941, **13,** 467–484.

CHAPTER 41

EXAMINATION OF MILK AND CREAM

Samples of milk and cream are examined when submitted by the staff of the Bureau of Milk Sanitation, and occasionally by other divisions of the Department. Reports are made to the Division of Sanitation. Health officers requesting the examination of milk to assist in carrying out the provisions of the Sanitary Code are referred to a local laboratory approved by the State Commissioner of Health for these examinations.

All samples sent to the laboratory must be accompanied by a record card on which are given the identification marks on the original container, the name and location of the dairy, bottling plant, creamery, producer, or distributor from whom the milk was obtained, the date and time of collection and of packing the sample, the grade of milk or cream, as raw or pasteurized, the examination required, and other pertinent information.

The methods of examination are, in general, those of the American Public Health Association and the Association of Official Agricultural Chemists (1).

Only representative samples are examined. They should be collected by sterile glass or metal tubes of a length sufficient to reach the bottom of the original container. All milk withdrawn should be put in screw-capped vials or glass-stoppered bottles protected against contamination and leakage. Vials and sample bottles should not be more than two-thirds full in order to permit proper agitation before portions are removed for examination. Bottled milk should be submitted in the original capped bottle as distributed by the dealer. Samples of milk and cream are shipped to the laboratory packed in sufficient ice to ensure constant refrigeration during shipment. Samples are not examined unless received at the laboratory with ice still present.

BACTERIOLOGIC EXAMINATION

Samples of milk are examined as soon as possible after receipt at the laboratory. Except during actual handling for purposes of examination, the samples are kept cold by means of cracked ice.

Standard Plate Count

Shake the sample vials vigorously with a vertical motion twenty-five times before removing portions for examination. Shake containers such as milk bottles twenty-five times in a vertical direction with alternate inversion of the bottle. Before opening the sample container, wipe the cap with gauze that has been soaked with 70-per-cent ethyl alcohol.

Plate dilutions of the sample in sterile tap water using beef-extract tryptone glucose skim milk agar (*F49B*). Plate at least two dilutions of each sample—a 1:100 and a 1:1000 for routine samples of good quality. With cream or low quality milk, plate 1:1000 and 1:10,000 dilutions. Use pipettes that have been certified by the director of the New York State Agricultural Experiment Station.

Incubate the plates for forty-eight hours at 35°–37°C.; invert during incubation to reduce formation of spreading colonies.

Make all counts in an illuminated counting device with a lens of 1.5 magnifying power. Count the plates that contain between 30 and 300 colonies. If it is necessary to count a plate with a greater number of colonies, select a representative fractional area bearing 150 to 200 colonies and multiply the count by the proper factor to obtain the number of colonies on the plate. If the plate contains less than 30 colonies, record only as found.

Take the plate count multiplied by the factor of dilution as the standard plate count per ml. of sample. If more than one plate from a given sample contains between 30 and 300 colonies, base the report on the average of their calculated results.

Results of the Test.—Report as the standard plate count per milliliter at 35°–37°C. Use only two significant digits in reporting the result.

Test for Bacteria of the Coliform Group

Inoculate each of three fermentation tubes containing brilliant green lactose bile medium (*F24*) with 1 ml. of sample. Incubate for forty-eight hours at 35°–37°C. Consider the production of gas in an amount occupying 10 per cent of the volume of the inner tube as evidence of the presence of bacteria of the coliform group in the portions tested. Consider lack of gas production or gas production of less than the prescribed amount as evidence of the absence of bacteria of the coliform group in the portions tested.

If, in special investigations of milk supplies, more precise information as to the density of coliform bacteria is required, inoculate a series of fermentation tubes containing brilliant green lactose bile medium with three portions of each of several submultiples of 1 ml. of the sample and incubate for forty-eight hours at 35°–37°C. Continue the test as above.

Results of the Test.—Report the density of bacteria of the coliform group as the most probable number (M.P.N.) per 100 ml.; compute the most probable number according to the tables of Hoskins (1, 2).

Direct Microscopic Count

The direct microscopic count gives valuable information as to the bacterial density of raw milk. It is not satisfactory for the grading of "Certified" or other grades of raw milk with low counts. It will, however, detect marked deviations from these grades, and is, therefore, a desirable supplementary procedure in their examination. Used in conjunction with the standard plate count of pasteurized milk, the direct microscopic count often discloses an unsatisfactory condition of the raw milk. Since the method may reveal the presence of flora introduced in the processing, it is used to supplement the standard plate count on pasteurized as well as raw milk samples.

Preparation and Staining of Films.—Prepare films on microscope slides, either 3- by 1-in. etched on one edge, or 2- by 4½-in. etched on both edges. Films from more than one sample may be put on one slide provided that all films can be dried within five minutes of the time of deposition of the sample. Mark the sample number on the etched portion of the slide with a lead pencil. Place the slide over a glass or paper guide ruled in square centimeters. Shake the container vigorously twenty-five times before removing portions for examination. Deposit 0.01 ml. of the sample on the slide with a standard pipette certified for the purpose by the director of the New York State Agricultural Experimental Station. The pipette and slides must be clean but not necessarily sterile. Spread the milk evenly over an area of 1 sq. cm. with a clean stiff needle, following the marking on the ruled guide. To speed the drying, place the slide on a level surface in a warm place. Do not heat excessively or the film will crack. After drying, dip the slide in recently filtered Newman-Lampert staining solution No. 2 (p. 38). Remove immediately, drain until thoroughly dry, and wash in distilled water. Dry and examine the stained film under the microscope. In the examination of samples of cream, first immerse the slide in xylol for from one to five minutes to extract the butterfat, then in 95-per-cent ethyl alcohol until the film is flattened, thin, and transparent. If droplets of butterfat are still visible on the film, repeat both the xylol and the alcohol treatments. Drain dry and proceed with the staining as described.

Counting.—Use a microscope fitted with an oil immersion objective and a selection of oculars that will provide fields of the proper diameter. Insert a micrometer disk in the ocular and adjust the draw-tube so that the diameter of the circle inscribed on the disk is either 0.146 or the diameter of the entire field is 0.206 millimeters. Standardization of the microscope with a stage micrometer is a necessary prerequisite for this adjustment. The field with a diameter of 0.206 millimeters will cover almost exactly the area occupied by 1:300,000 ml. of milk, and the field with a diameter of 0.146 millimeters will equal the area occupied by 1:600,000 ml. of milk.

For routine examination of milk samples, use a field diameter of 0.206 mm. and count all of the individual bacteria and clumps of bacteria observed in 30 fields. For purposes of this enumeration a clump of bacteria is considered to be either a single bacterial cell or a group of bacterial cells which in the judgment of the observer may have been produced from a single cell. Multiply the count by 10,000 to obtain the estimated clump count per milliliter. For a more accurate enumeration in milk of low bacterial count use a field diameter of 0.146 mm. and count all the clumps of bacteria observed in 60 fields. Multiply the count by 10,000 to obtain the estimated clump count per milliliter.

Make a separate enumeration of chains or clumps of chains of streptococci each of which contain 10 or more cells.

Make an enumeration of cells including epithelial cells and leucocytes.

Clean the slides with xylol, dry, and keep on file at least one month.

Results of the Test.—Report the number of bacteria and clumps of bacteria per milliliter as the direct microscopic clump count.

Report the number of long-chain streptococci per milliliter.

Report the number of epithelial cells and leucocytes per milliliter.

EXAMINATION OF DAIRY CONTAINERS

Containers used in the dairy industry must be free from bacterial contamination within practical limits, as defined in Standard Methods for the Examination of Dairy Products.

Samples to determine bacterial contamination are secured by the field staff of the Division of Sanitation by rinsing containers with buffered water (*F63.3B*). The procedures outlined in Standard Methods for the Examination of Dairy Products are followed.

All rinses are shipped to the laboratory in screw-capped vials or glass-stoppered bottles protected against contamination and leakage. The vials or bottles must be packed in sufficient ice to ensure constant refrigeration during shipment. Samples are not examined unless received at the laboratory with ice still present.

BACTERIOLOGIC EXAMINATION

Standard Plate Count

Before opening the sample container, wipe with gauze that has been soaked with 70-per-cent ethyl alcohol.

Plate the sample or dilutions of the sample in beef-extract tryptone glucose skim milk agar (*F49B*).

Plate 1 ml. of bottle rinses. Plate 1 ml. of can rinses, and in addition, 1 ml. of a 1:10 dilution in sterile tap water.

Procedure.—See "The Examination of Milk and Cream," p. 601.

Results of the Test.—Report the count as bacteria per milliliter of rinse water growing on agar within forty-eight hours at 35°–37°C. This value, multiplied by the number of milliliters of rinse water added to the bottle, may be considered as the number of colonies per container. Use only two significant digits in reporting the results.

DETERMINATION OF PASTEURIZATION OF MILK AND CREAM

Samples of milk and cream submitted for determination of pasteurization should be forwarded to the laboratory in cracked ice. If not more than twenty-four hours have elapsed between the time of collection and receipt at the laboratory, samples received uniced may be considered satisfactory for examination. To prevent souring during transportation of unrefrigerated samples, chloroform, in the amount of two drops for each 10-ml. sample should be added.

The Phosphatase Test (3, 4)

Buffer substrate.—Dissolve 1.09 grams of disodium phenyl phosphate and 11.54 grams of sodium veronal in water saturated with chloroform and dilute to 1 liter. Add 10 ml. of chloroform per liter and store in refrigerator.

Folin-Ciocalteu phenol reagent.—Dissolve 100 grams of sodium tungstate, $Na_2WO_4 \cdot 2H_2O$, (according to Doctor Folin), and 25 grams of sodium molybdate, $Na_2MO_4 \cdot 2H_2O$, in 700 ml. of water in a 1500-ml. flask connected by a ground-glass joint to a reflux condenser. Add 50 ml. of 85-per-cent phosphoric acid and 100 ml. of concentrated hydrochloric acid. Reflux gently for ten hours. Cool, add 150 grams lithium sulfate, 50 ml. water, and 4–6 drops of liquid bromine. Boil without a condenser for fifteen minutes to remove excess bromine. Cool, dilute to 1 liter with distilled water, and filter.

The finished reagent should have a golden yellow color. Reject any reagent with a greenish tint. Keep refrigerated and well protected from dust.

Dilute one volume of this stock solution with two volumes of water before use.

Sodium carbonate solution.—Prepare a 14-per-cent or 1.3 *M* solution of anhydrous sodium carbonate, Na_2CO_3.

Filter paper.—Whatman No. 40, 11 cm. in diameter, for the first filtration, or Eaton-Dikeman Company filter paper New Filt Nos. 1 and 3. (Interfering substances are at times extracted from other grades of filter paper.)

Pipette 10 ml. of buffer substrate solution into test tubes 20 by 160 mm. Add 0.5 ml. of the milk to be tested. Add a few drops of chloroform to inhibit bacterial action. Mix thoroughly by rotating the tube and warm to 37°–39°C. in a water bath. Cover to protect from dust. Paper toweling placed over the open end of the tubes has been found satisfactory. Do not use rubber or cork stopper. Incubate at from 35°–37°C. for not less than eighteen and not more than twenty-four hours.

After incubation cool and add 4.5 ml. of diluted Folin-Ciocalteu reagent. Mix and allow to stand for three minutes. Filter using either of the papers recommended for the first filtration and transfer 5 ml. of the filtrate to test tubes of 13-mm. inside diameter. Add 1 ml. of 14-per-cent solution of anhydrous sodium carbonate. Mix thoroughly by rotating the tube.

Place in a boiling water bath for five minutes and filter through any good grade of filter paper into test tubes of 13-mm. inside diameter. (Eaton-Dikeman Company paper Nos. 612 or 655 have been found satisfactory.) Evaluate the color of the filtrate by comparison with the permanent standards (see table 72) or by the use of a photoelectric colorimeter.

Samples of cream, particularly heavy cream, should be warmed to 37°C. to reduce the viscosity before measuring the 0.5 ml. necessary for examination. Similarly, with samples of milk or cream in which butter has formed, warming to 37°C. and thorough mixing will permit measuring the 0.5-ml. test portion.

TABLE 72

Determination of Pasteurization of Milk and Cream

Preparation of permanent phenol standards

PHENOL	COLOR SOLUTION		
	Gray*	Red†	Blue‡
mg./0.5 ml.	*ml.*	*ml.*	*ml.*
0.01	0.30	0.106	0.96
0.02	0.40	0.140	1.16
0.03	0.55	0.180	1.65
0.04	0.65	0.216	2.10
0.05	0.78	0.252	2.56
0.06	0.92	0.286	3.00
0.09	1.30	0.326	4.40
0.12	1.70	0.360	5.70
0.15	2.50	0.396	7.10

The amounts of color solutions given above are combined and diluted to 10 ml. with distilled water. Place standards in clear glass vials with an inside diameter of 13 mm. Seal with molded caps having acid resistant liners.

* Cobalt chloride ($CoCl_2 \cdot 6H_2O$) 31.9 grams, copper sulfate ($CuSO_4 \cdot 5H_2O$) 67.5 grams, nickel sulfate ($NiSO_4 \cdot 6H_2O$) 75.0 grams, concentrated hydrochloric acid 32 ml., and concentrated sulfuric acid 45 ml. dissolved in distilled water and diluted to 500 ml. at a temperature of 20°C.

† Cobalt chloride ($CoCl_2 \cdot 6H_2O$) 476 grams dissolved in distilled water and filtered. 100 ml. concentrated hydrochloric acid added and diluted to 1 liter at a temperature of 20°C.

‡ Copper sulfate crystals ($CuSO_4 \cdot 5H_2O$) 300 grams, and 20 ml. of concentrated sulfuric acid dissolved in distilled water and diluted to 1 liter at a temperature of 20°C.

Control Test.—A control test to check against the deterioration of reagents and to determine the presence of substances in the milk which may interfere with the accuracy of the procedure should be made according to the method outlined below. The control value is usually very low—

0.01–0.02 mg. per 0.5 ml. of sample—and in such instances it need not be subtracted from the phenol value found in order to determine the concentration equivalent to pasteurization treatment.

Make the control test on all samples found underpasteurized and if the value is abnormally high, subtract the amount in excess of 0.02 mg. from the phenol value as determined and consider the difference as the concentration indicative of treatment. Since a high control value often indicates a possible error in technic or an unsatisfactory condition of the milk, additional samples from the same source should be examined if these are obtainable.

To 10 ml. of buffer substrate solution add 4.5 ml. of diluted Folin-Ciocalteu reagent and then 0.5 ml. of the sample milk. Do not incubate. Mix thoroughly by rotating the tube. Allow to stand three minutes. Filter and to 5 ml. of filtrate add 1 ml. of 14-per-cent sodium carbonate solution. Heat in a boiling water bath for five minutes. Filter again and read color with permanent color standards, using 13-mm. tubes.

To determine whether the buffer substrate reagent has deteriorated, make this control test occasionally, using the reagents but without adding the milk. The color obtained should not be greater than 0.01. If a higher reading is obtained, discard the reagent.

Results of the Test.—A phenol value of 0.045 mg. phenol or less per 0.5 ml. of sample has in general been found indicative of milk heated to 143°F. for thirty minutes. Values greater than this indicate progressively inadequate heat treatment. In reporting results give the milligrams per 0.5 ml. of sample as well as an interpretation that the milk is considered pasteurized or underpasteurized. Use the following as a basis for interpretation:

Mg. phenol/0.5 ml. sample	*Interpretation*
less than 0.05	pasteurized
0.05 to 0.14	underpasteurized
0.15 or greater	grossly underpasteurized

BIO-ASSAY OF VITAMIN D MILK

Chapter III of the State Sanitary Code requires that milk sold as containing vitamin D must have at least 135 international units per quart as determined by semiannual bio-assays made in a laboratory approved by the State Commissioner of Health. The technics are based upon the method given in "Methods of Analysis" of the Association of Official Agricultural Chemists (5). Either a 7-day or a 10-day assay period should be used. In place of the line test, X-ray photographs of the leg bones may be substituted. Before being photographed, the leg bone should be dissected and the skin removed, but it is not necessary to remove the muscle tissue from the bone. The type of X-ray equipment used should provide a clear definition when the bone is photographed without removal of the muscle tissue. A tolerance of not more than 0.2 in comparing the line test of the test animal with the control is permitted in determining the number of units in the sample under examination. The laboratories making bioassays of vitamin D are required to submit to this Division a copy of the report, which is transmitted to the Bureau of Milk Sanitation. Samples of vitamin D milk are purchased by the district milk sanitarians in the open market semiannually. The sampling dates are not known to the dairy.

REFERENCES

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(2) United States Public Health Service. Most probable numbers for evaluation of coli-aerogenes tests by fermentation tube method; by J. K. Hoskins. Washington, D. C., Government Printing Office, 1940. 29 p. (Reprint No. 1621 revised from Pub. Health Reports, 1934, **49,** 393-405.)

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CHAPTER 42

EXAMINATION OF SAMPLES CONCERNED WITH RESTAURANT SANITATION

These examinations are not made routinely but only in cooperation with the Division of Sanitation in special investigations. Health officials desiring examinations of this type are referred to a local laboratory approved for these procedures.

UTENSILS

At least three articles of each type, including glasses, cups, spoons, and forks should be selected at random, preferably from storage shelves. For a direct check on the efficiency of washing, however, utensils should be selected immediately on completion of the washing process. Adequate precautions should be taken to avoid contamination by handling during inspection.

A separate cotton swab should be used for each utensil. The swab should be moistened in a freshly opened vial of buffered water (*F63.3A*) and rubbed slowly over the significant surfaces of the utensil; the outer and inner rims of glasses and cups, the entire outer and inner surfaces of the bowls of spoons and the tines of forks. The swab should be replaced in the vial and the stick broken off just above the cotton by using the edge of the vial as a fulcrum. The samples should be refrigerated by cracked ice immediately after collection and transported to the laboratory by messenger.

All samples received at the laboratory must be accompanied by a record card on which are given the name and location of the establishment, the date and time of collection, and pertinent information regarding the identity of the utensils and the conditions under which they were washed and stored.

BACTERIOLOGIC EXAMINATION

Samples are examined as soon as possible after receipt at the laboratory Except during actual handling or shaking the samples are kept cold by means of cracked ice. Before examination the sample containers are shaken by hand until the cotton is fluffed out using a vertical motion. They may be shaken for five minutes in an automatic shaking machine that provides shaking on the vertical axis of the tubes.

Standard Plate Count

Before opening the sample container, wipe the cap with gauze that has been soaked in 70-per-cent ethyl alcohol.

Plate 1 ml. of the sample on beef-extract tryptone glucose agar *(F49C)*. Incubate the plates for forty-eight hours at 35°–37°C.; invert during incubation to reduce formation of spreading colonies.

Make all counts in an illuminated counting device with a lens of 1.5 magnifying power. Count all the colonies on the plate if the total number is less than 300. If the plate contains more than 300 colonies, select a representative fractional area bearing 150 to 200 colonies and multiply the count by the proper factor to obtain the number of colonies on the plate. Multiply the count by 10 to obtain the total colony count per utensil.

Results of the Test.—Report as the bacterial colony count per utensil, growth on agar within forty-eight hours at 35°–37°C. Use only two significant digits in reporting the results.

Test for Bacteria of the Coliform Group

The presence of bacteria of the coliform group on washed utensils is indicative of faulty methods of washing, handling, or storage; their absence cannot be considered in any case as evidence to the contrary.

Inoculate one fermentation tube containing brilliant green bile medium (*F24*) with 1 ml. of the buffered water suspension. Incubate for forty-eight hours at 35°–37°C. Consider the production of gas in an amount occupying 10 per cent of the volume of the inner tube as evidence of the presence of bacteria of the coliform group in the portion tested. Consider lack of gas production or gas production of less than the prescribed amount as evidence of the absence of bacteria of the coliform group in the portion tested.

Results of the Test.—Report the presence or absence of bacteria of the coliform group on the original utensil on the basis of the test.

WASH AND RINSE WATERS

This examination is not essential for the complete evaluation of cleanliness of eating, drinking, and cooking utensils, but it provides data that may be of value in accounting for the sanitary condition of the utensils and thereby serve as a basis for improvements in methods.

Samples of wash and rinse water, at least 100 ml., should be collected in sterile glass-stoppered bottles with a sterile pipette or similar device directly from the tanks while washing operations are in progress.

Samples should be refrigerated by cracked ice immediately after collection and transported to the laboratory by messenger.

BACTERIOLOGIC EXAMINATION

Samples are examined as soon as possible after receipt at the laboratory. Except during actual handling the samples are kept cold by means of cracked ice. Shake the sample bottles vigorously twenty-five times before removing portions for examination. Make standard plate count and report as on p. 610.

DETERMINATION OF PRESENCE OF DETERGENT

This test is useful in checking the concentration of the detergent in the wash water and to ensure the maintenance of satisfactory washing conditions. It is of particular value in studying the performance of dishwashing machines with respect to dilution of the wash water and carry-over of the wash water to the rinsing tank.

Titrate a measured amount of the sample with 0.02 *N* sulfuric acid using methyl orange as an indicator and calculate the alkalinity as calcium carbonate in parts per million. Titrate also a specimen of detergent solution prepared by dissolving the recommended amount of the detergent in clear water from the same supply used in the washing operation; calculate the alkalinity as calcium carbonate in parts per million.

Results of the Test.—Report both the alkalinity of the test sample and the alkalinity of the detergent solution of the recommended concentration, indicating that the concentration of a detergent solution is roughly proportional to its total alkalinity.

METHODS USED IN THE ANTITOXIN, SERUM, AND VACCINE LABORATORIES

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METHODS USED IN THE ANTITOXIN, SERUM, AND VACCINE LABORATORIES

INTRODUCTION

The period since the publication of the second edition of Standard Methods has witnessed notable changes in the practice of medicine as it relates to the prevention and cure of infectious diseases based on developments in medical laboratory science. These trends have been reflected in the activities of this department. Thus, production of antipneumococcus sera in the rabbit was further extended to include all of the different types with added stress on procedures for typing and standardization, while production of horse serum was reduced to three types. These in turn have now been largely superseded as therapeutic agents by the sulfonamides and more recently by penicillin, and the production of therapeutic antipneumococcus sera is, therefore, being discontinued. The use of antimeningococcus and antistreptococcus sera has also been greatly reduced for similar reasons. Nevertheless, it has seemed important to include in the present edition the procedures that have been employed routinely in the preparation of all these products and to record the changes that were adopted in technic. Reports of instances in which optimal results could only be obtained by a combination of chemotherapy and serum therapy, of drug sensitivity, and of drug-resistant bacterial strains, suggest caution before discarding completely such effective and complementary therapeutic agents.

Some entirely new methods or important changes in procedure are described. The principal new sections are those on the preparation of fluid tetanus toxoid, precipitated diphtheria-tetanus toxoid, enzyme concentration of antitoxic sera, and the preparation and standardization of phase I pertussis vaccine. Important revisions have been made in the sections on the standardization of antimeningococcus serum and typhoid vaccine, and the classification of strains of the meningococcus. The technical steps connected with the processing of human blood plasma and derivatives that is shortly to be undertaken are omitted since they are not yet a routine procedure. This also applies to Anti-Hemophilus influenzae Type b serum.

The work involved in the production, standardization, and preparation for distribution of the various antitoxins, sera, and vaccines, the diagnostic preparations, and related research, is divided among several groups, each responsible for the procedures associated with one or more usually related products.

The staff responsible for the animals at the laboratory and the laboratory farm, while actually representing a general service group, are especially associated with the activities of the Antitoxin, Serum, and Vaccine Laboratories and form a part of the department. Their chief activities, under expert veterinary supervision, include the care and breeding of laboratory animals, the care and treatment of horses under immunization, the prevention and study of epizootics, and the raising of farm produce used in the maintenance of the animals.

Because of its close relationship with physicians and the custodians of the district laboratory supply stations in the state, this department has advisory supervision of the distribution of all preparations and of the operation of the stations.

In addition to the various products prepared by the laboratory, certain others are purchased. These include silver nitrate solution, arsenical and bismuth preparations, sulfonamide drugs, penicillin, and certain sera and vaccines of which the amount distributed is small and restricted. Smallpox vaccine is purchased for distribution to clinics and for the vaccination of individuals to whom it would not otherwise be available.

Until the reactions of infection and immunity are more clearly understood, work based on their practical application must be largely empirical. Technical details in the methods of the Antitoxin, Serum, and Vaccine Laboratories are, therefore, constantly under revision and an entire procedure is subject to change. The routine methods can give at most only a cross section of a particular stage in the development of a problem. This state of flux makes uniformity of presentation impossible since the balance between precise directions and information or explanation necessarily varies with the data available for each process. It makes doubly important, however, a detailed statement of present procedure as a point of departure for future progress.

The aim has been to describe the essential technic and procedures so that they may be followed without difficulty. The minute details and instructions of local bearing only, which are available to the staff of the department as an operating manual, have been omitted as far as practicable.

CHAPTER 43

GENERAL INSTRUCTIONS

Records and Record Forms

Careful and complete records are kept of all procedures in both routine and experimental work. Enter each step as soon as data are available on the permanent record forms provided for the purpose. When recording experimental procedures for which no forms are provided, use 4- by 6-inch cards. Make the original entries directly on the permanent forms. If this is impracticable, do not destroy the original record until the copied entries have been compared by a second person and a sufficient time has elapsed to insure that the permanent record contains all essential data. In procedures that involve calculations, such as dilution of vaccine, concentration of antitoxin, and standardization tests, make the original calculations on the backs of the record cards or sheets or on a separate card to be filed with the test record, and have them checked by a second worker before the work is commenced. Records of tests and of other procedures requiring it should be initialed by the worker performing the test and, when indicated, by the person checking the data. Records of experimental work are transferred to the library and publications department for the scientific file when studies are completed.

Aseptic Precautions

When procedures requiring asepsis are described in the text, it should be understood that the materials used, such as glassware, stoppers, and connections, were previously sterilized. The adjective "sterile" is used only when for clarity a distinction between clean and sterile equipment is desirable.

In general, carry on all procedures connected with products for human use, which require rigidly aseptic precautions, in small, specially designed rooms. Before entering the room, wash the hands thoroughly with soap and water and apply a 50-per-cent glycerol-alcohol mixture if the skin is dry or rough. Wear sterile gowns and caps. Avoid unnecessary passing in and out of the room.

Rooms for Rigidly Aseptic Procedures.—Always keep the doors of the rooms closed. If a room has not been used for two or more days, give it a thorough preliminary cleaning on the day preceding its use. Immediately before beginning work, wipe thoroughly the surface of the table with a cloth wrung out in 1-per-cent cresol. After finishing work, remove the apparatus and supplies, then wipe all flat surfaces, including the floor, similarly. As often as necessary, wash the windows and surfaces with soap and water. Then wipe approximately dry with a cloth wrung out in the cresol solution. Never permit dust to collect on any surface.

Gowns and Caps.—Gowns and caps wrapped in muslin bags or heavy paper are sterilized before use at 15-pounds pressure. Put them on immediately before entering the room and remove them on leaving it. Adjust the cap to cover the hair completely.

Cultures

The worker in charge of a group to which the care of standard strains has been assigned is responsible for their maintenance. Transfers are made only by a trained, responsible worker. Rigid precautions must be taken to avoid mixing the cultures. As far as possible the work with one strain should be completed before that with another is commenced.

Label every culture with a gummed label so placed on the tube that it will not interfere with the observation of growth. Give the kind and number of the culture and the date of transfer. In general, when a culture that is to be retained is opened, it is advisable to enter on the label the date of opening and, when indicated, the initials of the worker. When paraffin-sealed plugs are used, reseal the plugs after a transfer.

Material in Bulk

Close all bottles that contain sterile material in bulk with rubber stoppers previously boiled in 0.5-per-cent cresol and dried in the flame, cover with cotton dampened with 1-per-cent cresol, tie in place, and cap with a suitable covering. When a bottle is to be reopened, remove the covering and wipe the stopper and top of the bottle with the cresol solution.

Number lots of the same kind of material in bulk consecutively; if a lot consists of more than one bottle designate each bottle with a letter (if one bottle, 66, if two, 66A and 66B respectively). If the same lot consists of two or more comparative batches, use Roman numerals to distinguish the batches (66I and 66II). If part of a lot, batch, or bottle is taken for further treatment distinguish the treated material by Arabic numerals in parentheses. Thus, if only one portion of 66A is treated, designate it as 66A(1), if both, as 66A(1) and 66A(2) respectively. If the contents of 66A are dispensed in containers of different sizes, designate the larger as 66A-1, the smaller as 66A-2; if dispensed at different times 66A-a and 66A-b respectively.

Identify plasmas and sera to be used for concentration by the horse or rabbit number and the bleeding numbers (H426–8) until pooled for concentration. Then designate each pooled lot of the same kind of material by a serial concentration order number. After filtration of the concentrated material, give each such lot a final product number. Follow the scheme described above for different bottles and comparative batches.

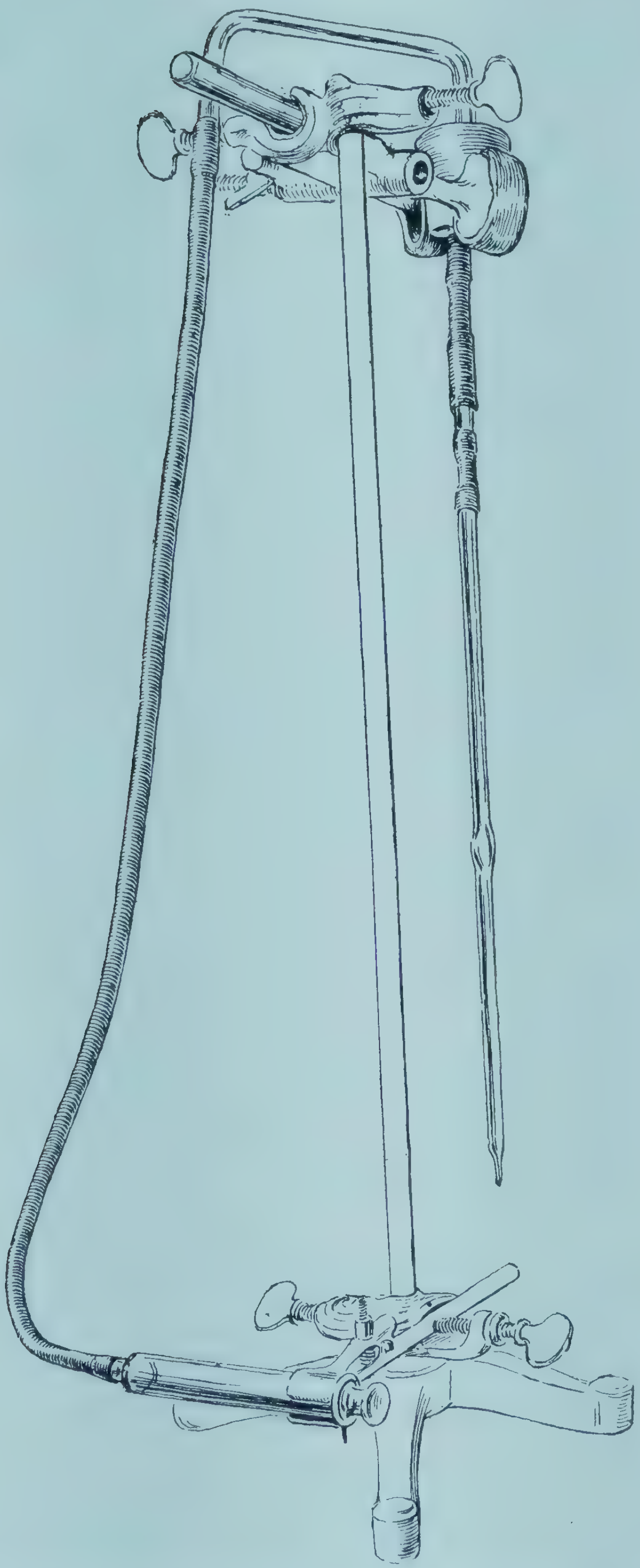

FIG. 63. APPARATUS FOR FILLING "TO CONTAIN" PIPETTES

Special Apparatus and Supplies

Apparatus for Filling "To Contain" Pipettes.—The stand for filling and emptying "to contain" pipettes consists of (1) an iron ring stand with tripod base and rod 26 inches high; (2) a small clamp attached to the foot of the rod which holds a 5-ml. Luer syringe with an air-tight plunger coated with a mixture of beeswax and vaseline (*F80*); (3) a large clamp attached to the top of the rod which supports an 8-inch horizontal glass tube with both ends bent downward 3 inches; (4) rubber tubing which connects one end with the syringe; (5) a short piece of rubber tubing for holding "to contain" pipettes, attached to the other end. This apparatus holds the pipette in a position that enables the operator to see the graduation clearly and to draw the material exactly to it. (See fig. 63.)

Sterile Siphon and Burette with Stopcocks and Side Arm.—Assemble a 2-holed rubber stopper with an air-inlet tube and a glass siphon with rubber connections, the ends of which are protected by cotton pads or paper caps. Wrap the apparatus in paper and sterilize by autoclaving. Clean previously the glass connections in a suitable detergent solution, see p. 246, the rubber stopper and connections by boiling in cresol solution (*F75*) diluted with an equal amount of water or in distilled water, and rinse thoroughly. To prepare the burette, fill it with either a suitable detergent solution or cleaning solution (*F78A*), allow to stand for at least one-half hour, then rinse thoroughly with tap and finally with distilled water. Fill it with 70-per-cent alcohol, make sure that the alcohol penetrates between the key and the bore of the stopcock, and cover the top of the burette with a sterile cap. The next morning, discharge the alcohol, plug the burette with sterile cotton, dry the stopcocks, key, and bore with sterile absorbent paper and cotton swab and smear with a sterile beeswax-vaseline mixture (*F80*). With aseptic precautions, connect the side arm of the burette with the siphon placed in the bottle of material to be dispensed. If drops adhere to the walls of the burette or the stopcocks stick, when the apparatus is used repeatedly, attach a pinchcock to the rubber tubing between the siphon and burette, disconnect the burette, insert the end of the tubing into a sterile tube, and clean the burette as previously described. From time to time dismantle and clean the entire apparatus.

Method of Dispensing from Burette.—When a solution is to be dispensed, wipe the tip of the burette with cotton moistened with alcohol. Always discard a few drops of the solution before the material is brought to the desired graduation mark. Wipe the tip of the burette with sterile absorbent paper. Hold the flask so that the tip of the burette extends below its neck. Dispense the required amount and touch the tip of the burette to the inside of the flask in case a drop has adhered. A meniscus reader placed with the black edge 0.1 ml. below the selected graduation may be used to facilitate readings. When the burette is not in use the level of the solution should always be above the zero mark to guard against the formation of a film or crystals in the graduated area.

Closed System of Siphoning.—To transfer fluid material from one bottle to another with aseptic precautions, when the use of a pipette is not practicable, assemble a siphon apparatus as follows: For the bottle containing the material, prepare a stopper with a siphon-outlet tube and an air-inlet tube, which may be connected with a Woulff bottle or protected by a cotton and cheesecloth pad that also serves as an air filter. For the bottle to receive the material, equip a second stopper with an inlet tube and an air-outlet tube provided with a cotton guard conveniently made from a discarded bulb pipette. Use rubber tubing to connect the siphon-outlet tube in the first stopper with the inlet tube in the second. Wire all glass and rubber connections. Protect the stopper and the long end of the siphon that is to be inserted into the bottle containing the material with a paper envelope; the second stopper and glass connections to be inserted in the receiving bottle, with a cotton and cheesecloth pad. Wrap the entire apparatus in heavy paper for sterilization.

To set up the apparatus, insert the stoppers in the bottles with aseptic precautions. Secure the stoppers with a light cord tied around the neck of the bottle and across the top of the stoppers. Wrap them with strips of cotton dampened with 1-per-cent cresol. If the gravity method is to be used, apply vacuum to the second bottle only to start the flow; if not, use continued vacuum.

For washing precipitates under aseptic conditions, prepare two stoppers as for the closed system, one for the bottle of washing fluid, the other for the waste bottle. Connect them with a 3-holed stopper (to be used in the bottle containing the precipitate) prepared with (1) a siphon-outlet tube to be connected with the waste bottle, (2) an inlet tube to be connected with the siphon of the washing bottle, and (3) an air tube protected by a cotton guard. One part of the system should be closed by a pinchcock when the other is in operation.

Syringes.—All new syringes are tested for leakage around the piston and at the junction with the needle. Those to be used for the accurate measurement of volumes in special work are tested also by measuring in an accurately graduated "to contain" cylinder. For more precise methods of calibration, see p. 129.

Pipettes.—For the cleaning and sterilization of pipettes for general use, see "Preparation of Glassware," p. 247. Specially standardized pipettes that are used for non-infectious material are cleaned by the workers handling them. Place each pipette as used in tap water. When the test is completed, rinse the pipettes thoroughly in running water and place in cleaning solution (*F78A*) for at least one-half hour. Rinse thoroughly in tap water followed by distilled water. Draw up alcohol (previously used for final rinsing) twice, then 95-per-cent alcohol once. Dry the pipettes in a slanting position. Unless the pipette is used within a day, protect the tip in a plugged tube with a cotton pad at the bottom; certified pipettes with similar tubes at each end.

Small Cotton Filters.—To prepare the cotton filters often used in filtering small volumes of material such as culture-suspensions, select short pieces of glass tubing of large bore (length 180, diameter 19 or 25 mm.). Draw out the tubing so that the middle section is from 3 to 5 mm. in diameter and from 100 to 150 mm. long. File and break in the center. Anneal the broken tips in a flame. Insert the filter of absorbent cotton, plug the large end of the tube with cotton, wrap in paper, and subject to dry sterilization.

Salt Solution.—"Salt solution" as used in the text refers to a sterile 0.85-per-cent sodium chloride solution.

Cold Rooms.—Cold rooms are maintained at a temperature of from 4°–6°C.

CHAPTER 44

PRODUCTION AND STANDARDIZATION OF DIPHTHERIA TOXIN

Diphtheria toxin is prepared and standardized for the production of antitoxic serum in horses, for standardization tests of unconcentrated and concentrated sera, for the intracutaneous test of susceptibility to diphtheria toxin (Schick), and for the preparation of toxoids for the active immunization of persons against diphtheria. The toxin is standardized by subcutaneous tests in guinea pigs. The flocculation test is also used to determine potency.

Strains

The standard strain used routinely in the production of toxin is:

Corynebacterium diphtheriae: Park-Williams No. 8. Collection No. 5 (original No. 110). Received in 1915 from the Bureau of Laboratories, New York City Department of Health, where it was isolated in 1894.

Another strain of *C. diphtheriae*, Collection No. 3203, isolated in this laboratory in 1930, is also used (1, 2).

Maintenance.—Two cultures of the standard strain are maintained independently since changes in toxigenic activity may occur. The cultures are grown on 0.2-per-cent glucose veal-infusion broth (*F12*); the toxigenicity is more stable in this medium. A loopful of the pellicle is transferred daily to the surface of 2 ml. of medium in a 125- by 13-mm. tube. The tube of culture is incubated at approximately 34°C. for 24 hours in a slanting position (an angle of about 20°). Parent cultures are retained for one week at room temperature. Two cultures of strain No. 3203 are maintained on synthetic medium (*F29.4*) by transfers every other day.

Each new lot of medium is tested before use for its ability to support satisfactory growth of *C. diphtheriae* in two successive transfers.

Purity Tests.—Before removing the cultures from the incubator room, examine for characteristic appearance of pellicle and sparkling clearness of broth. If the purity of a culture is questioned, make immediately duplicate microscopic preparations stained with alkaline methylene blue and by Gram's method, plate cultures by spreading a loopful of broth and pellicle over the surface of 0.2-per-cent glucose beef-infusion agar (*F35A*), and transfers from previous seedings for possible substitution. A somewhat cloudy broth may indicate a preponderance of short forms and a decrease in toxigenicity. Make microscopic examinations and plate cultures weekly.

Should a culture be contaminated, make transfers from previous seedings. Resort to fishings only if the strain would otherwise be lost.

PRODUCTION OF TOXIN

Infusion-free peptone medium (*F20*) is used routinely with the Park-Williams No. 8 strain for toxin production. Occasionally a lot of toxin is produced in veal broth (*F12*) to ensure a more stable material for use in the standardization of antitoxins and in the intracutaneous test.

Two modifications of the infusion-free medium (3, 4) differing only in their sources of energy are used:

1. Sodium acetate and maltose (*F20*, Solution I) in a sterile filtered solution added to the sterilized medium to give concentrations of approximately 1.25 per cent and 1.0 per cent respectively.
2. Sodium lactate and maltose (*F20*, Solution II) added similarly to give concentrations of from 0.7 to 0.9 per cent and 0.75 per cent respectively.

Glucose to give a final concentration of 0.05 per cent may be added in addition to the maltose and acetate or lactate. Experience over a period of several years has indicated considerable variation in the potency of each type of infusion-free toxin, due possibly to differences in some of the chemicals used. Toxins of the highest titers are at present produced in the maltose-sodium-acetate medium.

Strain No. 3203 has produced highly potent toxin in the infusion-free maltose-sodium-acetate medium. The sodium-lactate-maltose medium has not proved satisfactory for toxin production with this strain. The variation and marked differences in the vegetative and toxigenic activities of the two standard strains in these media, and especially in synthetic media, indicate the subtle biologic relations that obtain in the cultivation of the diphtheria bacillus.

The culture is grown for seven or eight days in 2-liter Pyrex "diphtheria toxin bottles," Roux type, or 2-liter Erlenmeyer flasks containing from 200 to 300 ml. of medium. The containers are allowed to stand at room temperature for three or four days before the broth is inoculated, to permit the reabsorption of oxygen after sterilization. Usually from 100 to 150 are inoculated at one time.

Preliminary Transfers and Inoculation of Toxin Medium.—Two days before the medium for toxin production is to be inoculated, make from 6 to 10 extra transfers from the selected stock culture to tubes of freshly prepared medium and incubate at approximately 34°C. The next day make duplicate microscopic preparations from these tubes and stain as previously described. Reject any cultures showing atypical forms. From the remainder, inoculate for seed cultures half as many Pyrex tubes (125- by 18-mm.) of the medium as the number of bottles or flasks to be inoculated.

Examine the seed tubes before removal from the incubator room and discard any that do not have the characteristic appearance. Using the flame method to prevent contamination, pour the required amount of acetate- or lactate-maltose solution into each container of toxin broth and inoculate the medium with a loopful of seed culture. Usually one seed tube is used for two or three bottles or flasks. Incubate at approximately 34°C. for seven or eight days. Toxins prepared in veal broth are incubated for seven days. If the incubator is open frequently, protect the flasks from the light with a dark cloth. Do not disturb them during the period of incubation.

Addition of Preservative. Filtration.—Examine the growth before the bottles are removed from the incubator room. If the broth below the pellicle is not clear and sparkling, or if the appearance of the pellicle is unusual, make slide preparations and stain by Gram's method. If necessary, make a second preparation and stain for sporeforming microorganisms with steaming carbolfuchsin. Discard any contaminated culture. Unless the toxin is to be used for toxoid, in which case the toxin without preservative is filtered into graduated bottles, add 5-per-cent phenol solution to each container to give a concentration of approximately 0.5 per cent and mix thoroughly by rotating. Filter preferably on the same day; if filtration is delayed, cover the bottles with a dark cloth.

Pass the toxin broth through cotton and filter paper to remove the pellicle. Toxins prepared in veal broth are clarified by filtration through infusorial earth. Collect the clear toxin broth in large bottles (eight-liter) to ensure adequate pooling, then filter through a Mandler filter candle. Remove samples for potency tests from one of the bottles and make the required tests for sterility.

STANDARDIZATION OF TOXIN

Diphtheria toxin is standardized by determining its minimum lethal dose for guinea pigs. The potency is expressed in M.L.D. per milliliter. The number of L+ doses per milliliter is determined for toxins selected for a standard and for other toxins whenever desirable. The flocculation test is also used as a measure of potency (5–7). Standardization tests are made on each lot the day the toxin is filtered.

One minimum lethal dose is the smallest amount of toxin that, when injected subcutaneously, will kill a guinea pig weighing between 230 and 280 grams in ninety-six hours.

The L+ dose is the smallest amount of toxin that, when mixed with one unit of standard antitoxin and injected subcutaneously, will kill a guinea pig weighing 250 grams in ninety-six hours.

The flocculation value of a toxin is expressed in Lf per milliliter. Lf represents the amount of toxin that gives initial flocculation with one unit of antitoxin and is expressed as the reciprocal.

TEST FOR M.L.D.

Different amounts of the toxin to be tested are injected subcutaneously into a series of guinea pigs.

Special Glassware

The burettes and pipettes used for measuring the volumes must be standardized accurately and all glassware must be perfectly clean.

Burette.—50-ml. burette, certified by the Bureau of Standards, with a siphon attached. For cleaning and sterilization, see "General Instructions," p. 620.

Syringes.—3-ml. syringes (needle, 22 gauge, $1\frac{1}{4}$ inches) tested for possible leakage.

Pipettes.—"To contain" pipettes standardized in this laboratory (8) and graduated pipettes certified by the Bureau of Standards. The pipettes should be absolutely dry before use.

Dilution flasks and cylinders with glass stoppers.

Small amber bottles with wide necks, fitted with corks—$\frac{1}{4}$ ounce capacity.

The Test

Healthy guinea pigs weighing between 230 and 280 grams are used. Generally, three different doses of toxin are injected subcutaneously, 0.004, 0.003, and 0.002 ml., or 0.003, 0.002, and 0.001 ml., depending upon the potency of recent lots. One guinea pig is used for each dose.

Dilutions.—All dilutions are made in salt solution so that the desired amount of toxin is contained in not less than 0.5 ml. and not more than 2 ml. For example, if the doses to be injected are 0.003, 0.002, and 0.001 ml. prepare a 600-fold dilution and inject 1.8, 1.2, and 0.6 ml. respectively, see p. 24.

When all the flasks have been filled with salt solution from the certified burette, adjust a 1-ml. "to contain" pipette in the special filling apparatus, see p. 619. Draw up the toxin until the bottom of the meniscus just reaches the graduation. Should the toxin go up in the pipette no more than 1 mm. above the required graduation, draw it back by touching filter paper to the tip of the pipette. Should the toxin be accidentally drawn higher, use a fresh pipette. Wipe the tip of the pipette with filter paper, verify the reading, and deliver the contents into the first flask of salt solution. Replace the stopper and invert the flask gently two or three times. Draw up exactly 1 ml. to rinse the pipette, discharge the contents into the flask, and mix as before. Rinse again. With a fresh pipette draw up 1 ml. of the first dilution and discharge into the flask prepared for the second. Rinse twice. If more than two dilutions are to be made, continue, using a fresh pipette for each dilution.

When two or more toxins are to be tested, it is preferable because of the instability of toxin dilutions to complete each test, including the animal inoculation, separately. Otherwise, all of the first dilutions are made before any of the second.

When the dilutions are completed, transfer with a certified 2-ml. graduated pipette the required volumes to small amber bottles. Before emptying the pipette, wipe the tip with filter paper and verify the reading. Then touch the tip to the inside of the bottle to ensure an even flow, and discharge. With an ordinary pipette, add to each bottle sufficient salt solution to make a total volume of 3 ml. Rotate the bottle gently, taking care that no liquid touches the cork.

Injections.—Draw the contents of the first bottle into a 3-ml. sterile Luer syringe; pipette 1 ml. of salt solution into the emptied bottle and rinse by rotating carefully.

While the assistant holds the guinea pig, select as the site of injection an area slightly above and to the right of the umbilicus. Insert the needle subcutaneously, pointed away from the head and obliquely toward the median line, and inject the dose slowly. Draw up the rinsings into the syringe, and holding the syringe vertically with the needle upwards draw down the plunger to beyond the 3-ml. graduation to rinse. Expel the air carefully so that no drops of liquid are lost, and inject the contents on the opposite side of the abdomen. Watch the sites of injection and record any oozing that may occur. Use the same syringe for all doses. Begin with the smallest dose and rinse with salt solution between injections.

Observation of Animals.—Observe the animals daily at 9:00 a.m. and 5:00 p.m., more frequently if indicated. Record the degree of illness of each guinea pig. Place all dead animals in a low temperature chamber until the necropsies are performed. On the tenth day discharge animals that are still alive.

Necropsies.—Autopsy guinea pigs within twenty-four hours of death; when a Sunday or a holiday intervenes, within forty-eight hours. Record the lesions characteristic of diphtheria poisoning (the degree of induration at the site of injection and of congestion of adrenals and lungs, and the presence of pleural fluid) and any unusual conditions. If infection is suspected, make a slide preparation and streak a blood-agar plate (*F43A*) with heart's blood and material from the lungs and from any other organ as indicated.

Estimation of the M.L.D.—The least amount of toxin that kills a guinea pig in ninety-six hours is recorded as the M.L.D. of the toxin. If the animal that received the largest amount of toxin survives for more than four days, or if all die within seventy-two hours, repeat the test using a different series of doses. In estimating the potency, the symptoms and time of death of all of the test animals should also be considered.

TEST FOR L+ DOSE

Different amounts of the toxin to be tested are mixed with one unit of standard antitoxin and injected subcutaneously into a series of guinea pigs.

The Test

For standard antitoxin and test animals, see "Standardization of Diphtheria Antitoxin," p. 653.

Dilutions.—The dilutions are made in salt solution according to the procedures described for the test for M.L.D., see p. 626.

Serum.—Dilute the standard antitoxin so that one unit is contained in 1.0 ml.

Toxin.—Depending upon how close an estimation of the potency is desired, from three to six doses of toxin are tested. The doses in succession should be at least three-quarters of the preceding amount. Closer intervals, if practicable, are preferable. Dilute the toxin so that the desired amounts are contained in not less than 0.5 ml. and not more than 2 ml.

A toxin with an Lf value of 24 would be expected to contain 12 or more L+ doses per milliliter. Thus, the doses tested would be in a range of from 0.15 to 0.05 ml.

Procedures for the preparation of the toxin-antitoxin mixtures and the injection and care of the guinea pigs are the same as those given for the standardization of diphtheria antitoxin, see p. 655.

Estimation of Potency.—The smallest amount of toxin that mixed with one unit of standard antitoxin kills a guinea pig in ninety-six hours is considered the L+ dose of the toxin. For the determination of the L+ dose of toxin to be used as a standard toxin, see p. 654.

FLOCCULATION TEST

Varying amounts of the standard serum are mixed with a constant amount of the toxin to be tested. The tubes containing the mixtures are incubated in a water bath until flocculation occurs in one tube of a series.

The Test

Standard Serum.—A serum containing from 300 to 1000 antitoxic units per milliliter and showing well-defined flocculation within from twenty to thirty minutes with several different toxins is selected as a standard. It is standardized by repeated comparative subcutaneous tests with standard antitoxin from the National Institute of Health, see "Production and Standardization of Diphtheria Antitoxin," p. 654. When possible, comparative flocculation tests have been made with standard diphtheria antitoxin obtained from the Health Organization of the League of Nations. Since it has been found that titers obtained in subcutaneous and flocculation tests do not always agree, a serum that gives the same results by both methods should, if possible, be chosen for a standard. The serum without preservative is dispensed in 6-ml. volumes in 10-ml. bottles that are sealed with paraffin and stored at about −10°C. A sample is retested by the subcutaneous method at least every eighteen months.

Procedure.—Measure with 1.0- or 0.2-ml. pipettes the selected amounts of standard serum into a series of tubes (133- by 16-mm. of uniform diameter). The range of serum measurements depends upon the potency of the toxin to be tested and the degree of accuracy desired. Usually a test consists of a series of five tubes. Pipette into each tube 10 ml. of the toxin to be tested. Stopper the tubes and mix with a single, quick, up-and-down motion. Incubate in a constant-level water bath held at 40°C., with the water adjusted so that the liquid in the tubes is immersed to about two-thirds of its height.

Readings and Estimation of Titer.—Readings are made by artificial light against a dark background at appropriate intervals to observe initial flocculation.

The estimation of the titer is based on the amount of serum with which the initial reaction occurs. If flocculation occurs simultaneously in two tubes, an approximate estimate may be made; if in more than two or in the first or last of the series, the test must be repeated.

The titer is estimated from the following equation:

$$\text{Lf} = \frac{\text{ml. standard serum} \times \text{units per ml.}}{\text{ml. toxin}}$$

When very high-titered toxins are to be tested, it may be necessary to use a high-titered antitoxin as a standard serum.

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CHAPTER 45

PRODUCTION AND STANDARDIZATION OF TETANUS TOXIN

Tetanus toxin is used for the production of tetanus antitoxic serum in horses and for the preparation of toxoid for the active immunization of persons and horses. The toxin is standardized by subcutaneous tests in guinea pigs.

The methods described for the production of tetanus toxin are provisional and have not yet been adopted as standard. Media and strains different from those previously employed have been substituted and are under investigation with the aim of obtaining a more effective product.

All procedures are carried on in a three-room unit of the laboratory that can be entered only from the outside and that is equipped with an incubator, a cold room, and a large steam sterilizer. The special precautions for handling sporeforming microorganisms are observed (see p. 4).

Strains

The strains used at present in the production of toxin are:

Clostridium tetani: Collection No. 4343. Received from the Squibb Laboratories, New Brunswick, New Jersey, in February, 1943. Originally a transfer of strain Collection No. 298 obtained from this laboratory in 1941.

Clostridium tetani: Collection No. 43415. Received from Dr. J. Howard Mueller, Harvard Medical School, Boston, Massachusetts, in October, 1943. Originally a transfer of strain Collection No. 298 obtained from this laboratory in 1939 (1).

Other cultures also maintained are No. 298, National Institute of Health, Washington, D. C., S24, and the Pease-Research strain, No. 20 (original No. 263A). The latter was isolated about 1904 at the laboratory of the New York State Department of Health, Albany, and was used for the production of potent toxin for many years.

Maintenance.—Stock cultures of strain No. 4343 are maintained in semisolid veal-infusion agar (*F45B*) in 150- by 19-mm. tubes containing 15 ml. of medium; strains No. 298 and No. 20, in semisolid agar medium (*F45A*). Transfers are made at least every three months.

Strain No. 43415 is maintained by daily transfer (except Sunday) in beef-heart-infusion medium (*F13.1C*) and incubation in an atmosphere of carbon dioxide and hydrogen (2).

To prepare the semisolid agar transfers, first boil the tubes of medium in a water bath for fifteen minutes to expel the absorbed oxygen. Cool rapidly to about 50°C. by mixing cold water with that in the bath. Place one or two of the most recent agar cultures in cold water and heat sufficiently to soften the medium, not over 70°C. Insert a pipette with a gentle rotary motion and carefully remove sufficient culture, avoiding air spaces, which may cause the material to rise suddenly against the plug of the pipette. Observing the required precautions, transfer about 1 ml. of culture into each tube of fresh medium. Rotate the tubes to mix the contents, cool rapidly in cold water, and incubate at 35°–36°C. for seven days. If tests for purity are satisfactory, seal the tubes with paraffin before storing in the cold room. Two or more unopened cultures from each of the four preceding transfers are kept in reserve.

For the daily transfers of strain No. 43415 boil the tubes of broth medium for fifteen minutes and cool rapidly. Make a slide preparation of the previous day's transfer and stain by Gram's method. If the culture appears pure, pipette the smallest amount to ensure growth (0.1 ml. per 5 ml. of broth is usually adequate). Incubate overnight at 35°–36°C. in an atmosphere of carbon dioxide and hydrogen (2).

Purity Tests.—Examine slide preparations of each of the agar cultures stained by Gram's method. If desirable, make a second preparation and stain for sporeforming microorganisms with steaming carbolfuchsin, streak each culture on two beef-heart-infusion blood-agar plates (*F43A*), and incubate at 35°–36°C. for five days, one under aerobic conditions, the other in the hydrogen jar under anaerobic conditions, see p. 11. A culture of strain No. 43415 is plated similarly once a month. Examine the plates both macroscopically and microscopically. Fish suspicious colonies and transfer to semisolid-agar tubes and blood-agar plates for further study. If necessary, prepare a broth culture of the fishing, incubate for five days, and inoculate guinea pigs intramuscularly as a test of toxin production. Should contaminating microorganisms be found, discard the culture and substitute a pure culture of the same series. Go back to a preceding transfer if necessary. Resort to fishings only if the strain would otherwise be lost.

PRODUCTION OF TOXIN

Veal- or beef-heart-infusion broth with hog stomach autolysate (*F13.1A*) is used at present for the production of toxin by strain No. 4343 (3). The toxins usually contain from 20,000 to 40,000 minimum lethal doses and from 50 to 150 L+ doses per milliliter. Highly potent toxins containing 100,000 M.L.D. and from 500 to 700 L+ doses per milliliter, have been produced by strain No. 43415 in this medium containing reduced iron (*F13.1B*) (1, 4). Other strains have not proved so highly toxigenic in this medium.

Strain No. 4343 is grown for eleven days in 2-liter Erlenmeyer flasks containing 1600 ml. of medium; No. 43415, for seven days in the same amount of medium containing reduced iron. The flasks are rapidly cooled immediately after the final sterilization and the medium inoculated the next morning. Twelve to eighteen flasks usually constitute a lot.

Preliminary Transfers. Inoculation of Toxin Medium.—If strain No. 4343 is to be used for seed, two days before the toxin broth is to be inoculated make a transfer of a semisolid-agar stock culture (not more than six months old) to veal-infusion broth (*F13.1A*) containing chopped veal. Following the procedures described on p. 631, pipette approximately 0.5 ml. of the agar culture. Incubate for from 18 to 24 hours at 35°–36°C. The next day, if the culture appears satisfactory, make a sufficient number of seed cultures (1 tube for each 2 flasks to be inoculated) by pipetting 0.5 ml. of the culture into veal-infusion broth (*F13.1A*) to which $FeSO_4 \cdot 7H_2O$ has been added. If strain No. 43415 is to be used, make a sufficient number of seed cultures in broth (*F13.1C*) from the previous daily transfer the day before the toxin broth is to be inoculated. Incubate for from 18 to 24 hours at 35°–36°C., the latter in a jar containing carbon dioxide and hydrogen. Make slide preparations of each culture and stain by Gram's method. Reject any cultures showing atypical forms.

After final sterilization of the toxin medium place the flasks in a warm water bath and cool rapidly to 50°C. The following morning pipette 8 ml. of culture into each flask. Do not shake the flasks. Incubate the medium without iron for eleven days at 35°–36°C., that with iron for seven days.

Addition of Preservative. Filtration.—After rotating each flask to bring the growth near the surface, make slide preparations, stain by Gram's method, and examine. Discard any flasks that contain contaminated culture. Unless the toxin is to be used for toxoid, in which case the toxin without preservative is filtered into graduated bottles, add sufficient cresol to each flask to give a concentration of approximately 0.25 per cent. Add the preservative drop by drop with a rotary motion of the pipette to prevent the formation of large globules, and mix by rotating the flask. Cover the flasks with black cloth and leave at room temperature if the material is not to be filtered until the next day.

Pool the contents of the flasks and pass the toxin through infusorial earth. When necessary, pass the filtrate through a "preliminary" coarse-grained filter candle for further clarification, and then through a final filter. Take a sample from one bottle for standardization tests and make the required sterility tests.

STANDARDIZATION OF TOXIN

Tetanus toxin is standardized by determining its minimum lethal dose for guinea pigs. The potency is expressed in M.L.D. per milliliter. The number of L+ doses per milliliter is also determined.

One minimum lethal dose is the smallest amount of toxin that, when injected subcutaneously, will kill a guinea pig weighing between 330 and 380 grams in ninety-six hours.

The L+ dose is the smallest amount of toxin that, when mixed with one-tenth of a unit of standard antitoxin and injected subcutaneously, will kill a guinea pig weighing 350 grams in ninety-six hours.

Standardization tests are made on the toxin the day it is filtered.

The flocculation test as a measure of the potency of tetanus toxin is used while still under study as a supplementary test. For procedure, see p. 628. The flocculation value (Lf) of high-titered toxins (30 or more Lf) appears to bear a relatively constant relation to the number of L+ doses. A toxin that contains, for example, 600 L+ doses per milliliter will usually have a flocculation value of approximately 60 Lf. The results of flocculation tests of lower titered toxins are not so constant, even when the toxin is blended with a more potent toxin. A high-titered concentrated antitoxin is used in the test.

TEST FOR M.L.D.

Except where indicated, the procedures are the same as for the standardization of diphtheria toxin, see p. 626.

The Test

Healthy guinea pigs weighing between 330 and 380 grams are used. Generally, four different doses of toxin are injected subcutaneously, 0.0001, 0.00005, 0.000025, 0.00001 ml., or less depending upon the potency of recent lots. One guinea pig is used for each dose.

Dilutions.—All dilutions of tetanus toxin are made in peptone solution (*F60.3*).

After the test, pour the toxin dilutions and any water or salt solution used in rinsing the pipettes and syringes into a pail for sterilization. Fill all the special glassware with cleaning solution (*F78A*) and allow to stand for at least one-half hour before handling.

Observation of Animals.—Record daily the symptoms of tetanus as:

S—very slight: beginning contraction and rigidity of muscles of trunk felt on grasping it gently; slight curve of trunk to one side; stiffness of extremities.

SS—more definite: stiffness of extremities quite apparent; lateral deflexion of trunk and rigidity of muscles increased; slight intermittent tonic spasm on touching.

SSS—very marked: rigidity of legs extreme; head may be drawn back but animal still able to rise when placed on side; tonic spasm pronounced.

SSSS—whole body rigid: head thrown back; animal unable to rise when placed on side; tonic spasms.

Necropsies.—Examine the site of inoculation and the abdominal and thoracic cavities. Usually some congestion of the lungs is present.

TEST FOR L+ DOSE

With few exceptions, the procedures are the same as for the standardization of diphtheria toxin. Healthy guinea pigs weighing between 330 and 380 grams are used. The dilutions of toxin are made in peptone solution (*F60.3*). The range of dilutions prepared depends upon past experience. With different media and strains, toxins containing only 50 and as many as 700 L+ doses per milliliter have been prepared. The standard antitoxin (see p. 662) is diluted in salt solution so that one-tenth of a unit is contained in 1.0 ml.

Estimation of Potency.—The smallest amount of toxin that, mixed with one-tenth of a unit of standard antitoxin, kills a guinea pig in ninety-six hours, is considered the L+ dose of the toxin.

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CHAPTER 46

PRODUCTION AND STANDARDIZATION OF BOTULINUS TOXINS

Botulinus toxins of types A and B, which are most commonly associated with human cases of botulism, are prepared for use in the immunization of horses for the production of univalent antitoxic sera and in standardization tests of the sera.

All procedures for the maintenance of the cultures and production of the toxins are carried on in the separate three-room unit of the laboratory. The special precautions for handling sporeforming microorganisms are observed (see p. 4).

Strains

The standard strains used in the production of toxin are:

Clostridium botulinum, type A: Strain A 16. Collection No. 109 (original No. 175 G). Received from the Harvard School of Public Health, Cambridge, Massachusetts, July, 1920. Isolated in 1919 from olives responsible for an outbreak of botulism in Alliance, Ohio.

Clostridium botulinum, type B: Nevin strain (1). Collection No. 14 (original No. 175 B). Isolated in 1914 at the laboratory of the New York State Department of Health, Albany, from cottage cheese responsible for fatal cases of botulism.

Two strains of *Cl. botulinum* isolated from imported fish associated with cases of botulism have been identified in the Branch Laboratory of this division as type E (2, 3). Toxins prepared from these strains have been used in the production of diagnostic rabbit sera.

Maintenance.—Stock cultures are maintained in glucose semisolid beef-infusion agar, *F36B*, (150- by 19-mm. tubes containing 15.ml. of medium). Transfers are made every three months. Heat the medium and cultures as for *Cl. tetani* (see p. 631). Using a protective mouthpiece, pipette 1 ml. of the most recent agar culture into a tube of the agar medium. Incubate at 35°–36°C. for seven days.

Purity Tests and Confirmation of Type.—Examine slide preparations of each culture stained by Gram's method. Streak each culture on two beef-heart-infusion blood-agar plates (*F43A*) and incubate at 35°–36°C. for five days, one under aerobic conditions, the other in the hydrogen jar under anaerobic conditions. For use of hydrogen jar, see p. 11.

The type of *Cl. botulinum* is confirmed by a subcutaneous test in guinea pigs on each lot of toxin at the time the potency test is made.

PRODUCTION OF TOXINS

Type-A and type-B toxins are prepared at different times. The cultures are grown for fourteen days in 900 ml. of veal-infusion broth (*F11*) in 1-liter Erlenmeyer flasks.

Preliminary Transfers. Inoculation of Toxin Medium.—Three days before the broth is to be inoculated, begin daily transfers of an agar culture to freshly prepared veal-infusion broth, *F11*, (175- by 22-mm. Pyrex tubes containing 30 ml. of medium). After the tubes of medium have been boiled and cooled, add sufficient 20-per-cent glucose solution to give a 2-per-cent concentration. Using a protective mouthpiece, pipette 2 ml. of culture for each transfer. Incubate at 35°–36°C. overnight. Examine slide preparations of each broth culture stained by Gram's method.

Cool the flasks of toxin broth, see p. 633. Add sufficient 20-per-cent glucose solution to give a 2-per-cent concentration and the contents of one tube of broth culture. Rotate the flasks gently and incubate at 35°–36°C. for fourteen days.

Addition of Preservative. Filtration.—Rotate each flask gently to bring the growth near the surface. Make a slide preparation from the growth in each container, stain by Gram's method, and examine. Discard any flask that contains contaminated culture. Pool those that are satisfactory and add 5-per-cent phenol solution to give a 0.5-per-cent concentration. The procedures for filtration are those given for tetanus toxin, p. 633.

Whenever the filtrate is to be used for the preparation of toxoid, filter without preservative.

STANDARDIZATION OF TOXINS

Botulinus toxin is standardized by determining its minimum lethal dose for guinea pigs. The procedures for the standardization of diphtheria toxin are followed except as indicated. At present the doses injected are 0.0008, 0.0004, 0.0002, 0.0001, and 0.00005 ml. A wide range of doses is tested since the different lots vary and, because the toxins are labile, it is desirable to make the estimate from the first test. Each lot is tested for confirmation of type by injecting one guinea pig with a mixture of 0.05 ml. of the toxin and at least 100 units of type-A antitoxin, and another with a similar mixture of toxin and type-B antitoxin. The animal that receives the heterologous antitoxin usually dies within a few hours, while the other shows no symptoms.

Record symptoms of the guinea pigs daily, as follows:

Sl. ill: slight weakness, flabby abdominal muscles.
Ill: symptoms increasing to general weakness and prostration.

Very ill: increasing prostration, emaciation, paralysis of the throat muscles, exuding saliva.

Dying: extreme prostration.

At autopsy examine especially the following:

Site of injection: usually no macroscopic changes. Occasionally slight infiltration or edema with or without hemorrhage.

Abdominal organs: general congestion with dark-colored liver and spleen (absent in acute intoxication).

Stomach: usually empty and distended.

Bladder: frequently distended.

Lungs: congested; may show large or small hemorrhages.

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CHAPTER 47

PRODUCTION AND STANDARDIZATION OF STREPTOCOCCUS TOXIN

Streptococcus toxins produced by the cultivation of selected strains of hemolytic streptococci in broth medium are used for the production and standardization of antistreptococcus serum, for the intracutaneous test of susceptibility, and for the active immunization of persons who the test indicates are susceptible to the toxin. The toxins are standardized by intracutaneous tests on animals and persons.

While the production of streptococcus toxin and antitoxin for the prevention and treatment of scarlet fever dates back many years to the early work of Moser (1) and Gabritchewsky (2), a renewed interest in the serum therapy and prevention of the disease followed the studies in 1924 of Dochez (3) and of Dick and Dick (4–6). The latter made use of the intracutaneous method of testing susceptibility as applied practically to diphtheria toxin by Schick (7) in earlier work. A streptococcus specific to scarlet fever, however, has not been differentiated (8–16). Scarlet fever can only be considered as one manifestation of streptococcus infection. The use of streptococcus culture filtrate, quite similar to those now employed in active immunization against scarlet fever, for treatment of streptococcus infection in the human subject, and the skin reactions following its use were reported in 1918 (17).

Strains

The standard strains are:

Streptococcus: Dochez N. Y. 5. Collection No. 165 (original No. 14 E). Received in May, 1924, from Dr. A. R. Dochez, Presbyterian Hospital, New York. Isolated from a case of scarlet fever, 1922.

Streptococcus: Collection No. 32369. Received in March, 1931, from Dr. A. F. Coburn, Presbyterian Hospital, New York, labeled R 18 (18).

Maintenance.—Strains in active use are maintained in beef-infusion broth (*F5A*) with passage through mice once a week. Pipette 0.1 ml. of the most recent heart-blood broth culture into a tube of broth, streak a blood-agar plate (*F43A*), and incubate at approximately 35°C. for from twelve to sixteen hours. Examine the plate culture and a slide preparation of the broth culture stained by Gram's method. If no contamination is indicated, dilute the culture. See "Production and Standardization of Therapeutic Antipneumococcus Sera," p. 684. Inject a mouse intraperitoneally with a lethal dose. At autopsy inoculate two tubes of broth and streak a blood-agar plate with a loopful of heart's blood. After incubation for from twelve to sixteen hours, if there is no evidence of contamination on the plate and in the slide preparations of the broth cultures, streak a blood-agar plate with each. Examine after incubation and, if pure, store the cultures in the cold room and retain for three months. Cultures from mice dying after forty-eight hours are not used.

Identification and Purity Tests.—Every third month, or more frequently if desirable, test the broth cultures made at autopsy for fermentation of lactose, mannitol, and salicin (*F5B*). For test, see "General Bacteriologic Technic," p. 17. Test also the toxigenicity of the strains and the neutralization of the toxins by control antitoxic sera.

When testing strains for toxigenicity and neutralization of the toxins, small amounts of filtrate, generally not more than 30 ml., are prepared. The inoculated broth is incubated at approximately 35°C. for forty-eight hours. The subsequent procedures are the same as those for the preparation of large lots of toxin. Toxins of the different strains are tested for potency in comparison with the homologous standard toxins, see p. 641. Neutralization tests are made with 5 skin test doses (S.T.D.) and sufficient univalent sera, produced with representative strains of different toxin groups, to neutralize 10 S.T.D. of the homologous toxins. The usual heated-toxin, broth, and serum controls are injected. See "Production and Standardization of Antistreptococcus Serum," p. 671. With new strains, two doses of the filtrate, 0.001 ml. and 0.0002 ml. contained in 0.1 ml., are tested as are also mixtures that contain these amounts of filtrate and sufficient sera of the representative strains to neutralize 10 S.T.D. of the homologous toxins. If desirable, further tests are made with smaller doses of toxin. Toxins are not usually tested in doses larger than 0.001 ml.

PRODUCTION OF TOXIN

For the production of toxin, the culture is grown for seven days in 700 ml. of beef-infusion broth (*F5A*) in 2-liter Erlenmeyer flasks.

By this method toxins with a potency of from 40,000 to 60,000 S.T.D. have been obtained with strain No. 165. Toxins that contain from 100,000 to 200,000 S.T.D. per milliliter have been produced with strain No. 165 by modifications of the standard method, which resulted in a richer growth (19). Apparently a definite relationship exists between the amount of growth of a given strain and the potency of the toxin produced. With the broth to which glucose in a 0.3-per-cent concentration and calcium carbonate (5 grams per 700 ml.) as buffer were added, toxins containing approximately 100,000 S.T.D. have been obtained after forty-eight hours' and seven days' incubation. Toxins of approximately 200,000 S.T.D. were produced after forty-eight hours' incubation in the same basic medium, prepared without sodium chloride, to which glucose in a 0.75-per-cent concentration and a phosphate buffer were added. The potencies of toxins prepared in broth dispensed in 700-ml. amounts in 2-liter flasks or in 3-liter amounts in 4-liter bottles are generally similar. These preparations have been found to be stable for at least two years. The results of studies of the toxins in connection with the production of serum have indicated, however, that the higher-titered toxins are not more effective than the less potent toxins as antigens, see p. 669.

Toxins prepared in 10-per-cent beef-infusion broth (*F5.5*) to which glucose and calcium carbonate were added have proved to be potent and are satisfactory for the immunization of horses.

Preliminary Transfers and Inoculation of Toxin Broth.—Two days before the broth is to be inoculated for toxin production make a broth culture from the latest heart-blood broth culture which has been tested for purity, streak a blood-agar plate, and incubate both at approximately 35°C. for from twelve to sixteen hours. Examine the plate culture and a slide preparation of the broth culture stained by Gram's method. If no contamination is indicated, pipette approximately 0.5 ml. into one or more small flasks of the toxin broth to which glucose to give a 0.02-per-cent concentration has been added, and streak a blood-agar plate. Incubate for from twelve to sixteen hours.

Examine for purity as previously described and if there is no evidence of contamination proceed with the inoculation of the flasks of toxin broth. Pipette sufficient sterile 20-per-cent glucose solution (*F62A*) to give a 0.02-per-cent concentration and, depending upon the volume of broth to be inoculated, from 1 to 5 ml. of the seed culture previously agitated to insure a uniform suspension. Incubate at approximately 35°C. for seven days.

Addition of Preservative. Filtration.—Examine the growth as the flasks are carefully removed from the incubator room. Streak a blood-agar plate and make a slide preparation from the growth in each container. Add 5-per-cent phenol solution to give an approximate 0.5-per-cent concentration and mix thoroughly by rotary motion. The following day examine the plate cultures and slide preparations stained by Gram's method, and discard any flask that contains contaminated culture.

In our experience the standard strain No. 165 produces a heavy, flaky growth which settles to the bottom of the container and leaves a clear supernatant. The growth of the majority of strains generally remains in suspension.

If the bacterial cells have settled, pass the clear supernatant in each container through cotton and filter paper; if they are in suspension, pass through infusorial earth in a Büchner funnel. Filter the pooled, clear material through a Mandler filter candle. Remove samples for the required tests for sterility and potency.

STANDARDIZATION OF TOXIN

Streptococcus toxins are standardized by comparing the reactions induced by them in intracutaneous tests on goats, rabbits, and persons with those of a standard toxin. The potency is expressed in S.T.D. per milliliter.

One skin test dose of toxin is the amount of toxin contained in 0.1 ml. which, when injected intracutaneously into persons known to be susceptible to the toxin, will induce a reaction equal to that induced on the same persons at the same time by the injection of 1 S.T.D. of the State and/or the Federal standard toxin.

Standard Toxin

The standard toxin is produced with strain No. 165 (Dochez N.Y. 5). The present toxin, which contains 45,000 S.T.D. per milliliter, was standardized on animals against both the State and Federal standard toxins and the results were controlled by tests on persons.

The toxin originally selected as a standard in this laboratory was prepared with the Dochez N. Y. 5 strain, and the potency was carefully determined on a large number of persons in comparison with the standard toxin of the National Institute of Health, which at that time was prepared with the Dick I and II strains. The present Federal standard toxin is prepared with the Dochez N. Y. 5 strain.

Toxins of representative strains of streptococcus used for the production of serum and for special studies have been standardized on rabbits and persons for use as the standard control toxins of these strains.

The variation in susceptibility of the different species of test subjects to streptococcus toxins of different strains makes it essential to have accurately standardized control toxins of the various strains, and a knowledge of the strain used in preparing the test toxin before estimating the potency. Rabbits susceptible to the standard toxin, strain No. 165, have been found to react to the toxins of other strains; persons are not so constant, however, one individual failing to react to a given toxin may react to another; another individual, the reverse. In general, however, the potencies of different toxins estimated from tests on rabbits have corresponded with those obtained in tests on persons when sufficiently large numbers were tested. This relation does not hold in tests on goats; toxins of certain strains estimated to have a lower potency than the standard strain, No. 165, on persons and rabbits, may have a higher titer when tested on goats. Unless the strain is known and a control toxin of the same group is available, goats are not used for titration of toxins; persons may be used, but a large number are required to obtain reliable results. Rabbits have proved the most satisfactory.

TESTS ON ANIMALS

White-skinned goats, preferably of Saanen breed, from about 1 to 4 years old are used. A preliminary test to determine susceptibility is made with 2 test doses containing 1 and 10 S.T.D. of the standard toxin, unheated and heated, and also with similar dilutions of uninoculated toxin broth containing 0.5 per cent of phenol. For general procedure, see "The Test." Goats that are not definitely susceptible to 1 S.T.D. of toxin or that react to the smaller doses of heated toxin or to broth are not satisfactory for standardization tests. During the period of the test the goat should be kept in a temperature of approximately 70°F.

Preliminary tests with the larger doses of heated toxin and uninoculated broth are essential, since frequently experimental toxins of lower titer than the standard are tested.

Goats are used in preference to rabbits if control toxins are available for the toxin group and especially when a large number of injections are to be made. The same animal may be used as long as the reactions induced by the standard toxin and the controls are satisfactory. An interval of rest of at least a month should be allowed between tests.

Rabbits with white skin (Flemish Giants, Chinchilla, Silver Fox, and Belgian) from 1 to 2 years of age, weighing 3000 grams or more, are also used for the standardization of streptococcus toxins. A preliminary test to determine susceptibility is made with 5 S.T.D. of standard toxin and the same amount of heated toxin for control; the broth control is omitted. Rabbits have not been found sensitive to the bacterial or broth proteins. Tests may be made on the same animal at monthly intervals as long as the susceptibility of the animal is satisfactory.

The Test

Usually 6 doses of the standard toxin are injected. The largest generally contains either 1 or 2 S.T.D.; the other doses, amounts successively decreased by 50 per cent. The aim is to obtain a graded series of reactions, from definite to slight, or no reaction. A similar number of doses of the test toxin are used. Heated-toxin controls are injected and in the case of goats a broth control also.

The variation in susceptibility of different animals to streptococcus toxins makes it desirable to use the large number of doses in standardizing toxins in order to insure an end point in the titration.

Preparation of Dilutions.—All dilutions are made in salt solution so that the desired amount of toxin is contained in 0.1 ml. See "General Bacteriologic Technic," p. 24. If the dose to be injected is 0.0001 ml., prepare a thousandfold dilution. After all of the containers have been filled from a burette (factory certified) with the desired volumes of salt solution, add the required amount of toxin to the first container with a 1-ml. graduated pipette. With a fresh pipette, mix the contents thoroughly by drawing up the solution and discharging it several times; transfer the required amount to the next container. With a different pipette, mix thoroughly the contents of any tube containing material to be used for a further dilution and with the same pipette transfer the required amount. Plan all dilutions so that not less than 0.5 ml. or more than 1 ml. will be pipetted.

Preparation of Heated-Toxin and Toxin-Broth Controls.—For the heated-toxin controls, pipette into tubes about 2 ml. of the smallest fold dilution of each toxin to be injected. Take care that no material touches any part of the tube that will not be below the water level of the bath. Immerse the tubes to at least two-thirds of their length in a water bath and boil for two hours. For the uninoculated broth control, prepare dilutions of broth to which phenol to give a 0.5-per-cent concentration has been added. Use a dilution equivalent to the lowest of any toxin tested.

Injections.—For preparation and intracutaneous injection of animals, follow the directions prescribed for goats on p. 71, for rabbits, on p. 76. With a 1-ml. tuberculin syringe and a 26-gauge needle, $\frac{3}{8}$ inch long, inject intracutaneously 0.1 ml. Use separate syringes for the toxin and for the heated-toxin and the broth controls. Between different doses of the same toxin rinse twice with the next dilution to be injected; between injections of different toxins, rinse twice with salt solution and twice with the next material to be injected.

Starting at the shoulder, 5 or 6 rows of from 12 to 14 injections approximately 5 cm. apart may be made on each side of the goat, the first about 7 cm. below the median line of the back; on the rabbit, 3 rows of from 7 to 10 injections approximately 4 cm. apart, the first just below the median line of the back. Slight differences in reaction may occur in the top as compared with the lowest rows, especially if too low. The doses of the standard control toxins are injected, whenever possible, in positions corresponding to those of the test toxins. If a number of test toxins are included in the test, the standard toxin is injected in a middle row. Since the center of each row, between the shoulder and the hip, is the most favorable for reading reactions, the doses of toxin that are expected to give the end point in the titration are injected into this region. The first series of injections in a row is therefore commenced with the heated-toxin control and followed by the largest dose of toxin. The second series in the same row is started with the smallest dose of the next toxin to be injected and ended with the control. The broth control may be injected wherever it is convenient.

Readings and Estimation of Potency.—Make readings in a bright light, on goats from eighteen to twenty hours after injection, on rabbits, from twenty to twenty-four hours. The reactions on the goat may fade rapidly after twenty hours. Record the degree of redness and swelling induced and the size of the reaction (vertical and horizontal diameters) in millimeters. Estimation of the potency of a toxin is based on a comparison of the reactions induced by it with those induced by the standard toxin. All reactions in a series are considered. The potency of the test toxin should be equal to or a multiple or fraction of the potency of the standard control toxin. If no end point is obtained in the titration, the test is repeated with smaller or larger doses of toxin as indicated.

TESTS ON PERSONS

In addition to the tests on animals, toxin selected for the standard, for the intracutaneous test of susceptibility, and for the active immunization of persons is standardized on persons of different degrees of susceptibility and persons known to be insusceptible. The reactions induced by the test toxin are compared with that induced by 1 S.T.D. of the State standard toxin injected at the same time. In tests of a new standard toxin, the Federal standard toxin is used also. Injections of the standard toxins and of the largest dose of the test toxin, heated, are made as a control. The flexor surface of the arm is generally used. See "Production and Standardization of Antistreptococcus Serum," p. 674.

The Test

Not less than 2 doses of a test toxin, based on the results of tests on animals, are injected. With aseptic precautions, prepare the dilutions and heated-toxin controls as for the test on animals.

Injections.—Cleanse the surface of the skin to be injected with soap and water and then with alcohol. Use 1-ml. syringes graduated in tenths, one for the toxins, another for the heated-toxin controls, and special 26-gauge needles, $\frac{3}{8}$ inch long, freshly sterilized for each person. Separate syringes and needles are reserved for human use only. Inject 0.1 ml. intracutaneously with aseptic precautions. Always commence a series of injections with the smallest dose. Inject test toxins on the left arm and standard toxins on the right, and place the corresponding doses of test and standard toxins and the heated-toxin controls in relative positions. For further details of the procedure, see "Tests on Animals," p. 644.

Readings and Estimation of Potency.—Readings are made in a bright light from twenty to twenty-four hours after injection. Record the degree of redness and swelling induced and the size of the reaction (vertical and horizontal diameters) in millimeters. In estimating the potency of a toxin all the reactions on each individual are considered. The amount of test toxin that induces a reaction equal to that induced by 1 S.T.D. of standard toxin is considered to contain 1 S.T.D. If all doses of the test toxin induce reactions equal to that induced by the standard toxin, repeat the test with smaller doses of the test toxin. The persons in whom the standard toxin induces no reaction should not react to the test toxin. A reaction may be induced by the test toxin if the potency has been underestimated or in the case of toxins of strains other than the standard.

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CHAPTER 48

PREPARATION OF SOLUBLE SPECIFIC SUBSTANCES OF THE PNEUMOCOCCUS

Pneumococcus soluble specific substances are prepared from culture filtrates for use in precipitation tests of therapeutic sera and in intracutaneous tests in connection with the serum therapy of pneumonia.

For the production of the specific substances of types 1 to 40, subtypes, and related types (1–3), pneumococci are grown in a medium prepared from peptone dialyzate (4, 5) (*F26*).

The use of such a medium facilitates the subsequent passage of the culture filtrate through the ultrafilter and a residue almost free from medium constituents is obtained. An even higher degree of purification can be secured if the dialyzate is ultrafiltered before it is made into medium.

After the addition of preservative to the culture, the microorganisms are removed in a Sharples supercentrifuge and the effluent is rendered clear and sterile by passage through a preliminary Mandler followed by a final Mandler candle. The concentrated crude soluble specific substance is finally purified by fractional alcoholic precipitations.

Variations in the growth of the different types of pneumococci are not sufficient to necessitate modification of the procedure for preparing and ultrafiltering the culture filtrate. The principles of the further purification are the same for all types although each preparation may require some modification of the details.

Preparation of Cultures

Use pneumococcus cultures suitable for the production of type specific therapeutic sera. From a 15- to 18-hour culture in peptone dialyzate medium (*F26*), in which has been incorporated 0.2 per cent of beet sugar, inoculate from twenty to thirty seed bottles (200-ml. French square bottles containing 100 ml. of the same medium). After incubation at 34°–36°C. for from five to six hours, inoculate with a 100-ml. pipette 3200 ml. of peptone dialyzate medium (*F26*) in a 4-liter bottle from each seed culture and streak a blood-agar plate. Also, with a pipette, add 100 ml. of 6-per-cent buffered beet-sugar solution (*F21*). Incubate at 34°–36°C. for from sixteen to twenty hours.

Addition of Preservative. Filtration.—Remove the bottles from the incubator, streak a blood-agar plate, make a slide preparation from each bottle, stain by Gram's method, and examine. Adjust the pH of each to approximately 7.0. Add phenylmercuric borate to give a concentration of 1:150,000 (1 ml. of a 0.67-per-cent solution per liter) and place in the cold room. On the following day examine the plates and discard any bottle that shows evidence of contamination. Pass the culture through a Sharples supercentrifuge and filter the effluent through a preliminary Mandler filter candle to clarify it further; then, with aseptic precautions, pass it through a final candle into 4-liter bottles.

It has been found convenient and satisfactory in such filtrations to invert the candle in a hydrometer jar containing the material that is to be filtered. The candle is connected by rubber tubing directly with the inlet tube in the stopper of the bottle that is to receive the filtrate. The tubing-candle connection is secured with copper wire. The bottle containing the unfiltered material is fitted with a 2-holed stopper, through one hole of which is passed a short outlet tube 8 cm. in length and through the other an air-inlet tube of smaller bore extending outside to within about one-half inch of the end of the outlet tube and inside nearly to the bottom of the bottle. So stoppered, the bottle may be inverted over the hydrometer jar in which the liquid will be maintained automatically at a constant level.

Mark the level of the filtrate in each bottle with adhesive tape in order to measure the exact volume after the bottle is emptied on the ultrafilter. Store the filtrates in the cold room until they are to be ultrafiltered.

Ultrafiltration of Culture Filtrates.—The material is passed with aseptic precautions through alumina thimble ultrafilters coated with 4.5-per-cent nitrocellulose (Parlodion), which retain the soluble specific substance. The filtrate is collected in 4-liter bottles; the contents of the first bottle from a filter are tested for the absence of a specific precipitation reaction with the homologous antiserum (0.05 ml. of serum and 0.1 ml. of filtrate). Any filtrate that gives a precipitation reaction indicating leakage of the filter is refiltered and the filter discarded if the leakage persists. From 15 to 20 liters of material are passed through a single membrane. The residue on the thimble is finally washed with sterile distilled water until the washings are colorless.

Purification of Soluble Specific Substances

The further purification of the soluble specific substance consists in a fractional alcoholic precipitation by which contaminating materials less soluble or more soluble than the type-specific substance are eliminated, leaving the latter in a middle fraction. All precipitations and centrifugations are carried out at 1°–6°C. The steps in the purification are controlled by precipitation tests with homologous immune serum and with heterologous antipneumococcus serum that reacts with the species carbohydrate.

When the residue (concentrated 100 times or more) is removed from the ultrafilter, note the volume and treat with three-quarters of this amount of alcohol, and chill thoroughly. Add a saturated solution of sodium acetate (adjusted to pH 7.0 with glacial acetic acid) until precipitation begins.

The addition of the electrolyte removes inactive material and, at the same time, leaves the soluble specific substance in solution. An excess at this point should be avoided since it may cause some of the soluble specific substance to precipitate.

Centrifuge the mixture at about 3000 r.p.m. for one-half hour. The major portion of the soluble specific substance remains in solution. In case an appreciable amount is carried down in the precipitate, it may be recovered by redissolving the precipitate in distilled water and reprecipitating with alcohol as described. Combine the second alcoholic supernatant with the first and treat with more alcohol ($\frac{3}{4}$ to $3\frac{1}{4}$ volumes per volume of water in the combined solutions). Add more sodium acetate solution, if necessary, to precipitate the main portion of the soluble specific substance. Do not use an excess since it may inhibit precipitation.

It is not practicable to define the conditions exactly since the additional amount of alcohol required to precipitate the soluble specific substance varies with the type of pneumococcus. Usually, $\frac{3}{4}$ to $1\frac{1}{4}$ volumes of alcohol are sufficient, although types 7A, 7C, 11, 15, 18A, 20, 22, 31, 39, and 40 may require slightly more, and type 4, considerably more. The concentration of soluble specific substance in solution also governs the amount of alcohol needed. It is desirable to use the least amount that is effective in order to avoid the precipitation of the more soluble "C" substance and other impurities. Make trial tests with small aliquots before treating the bulk of the material.

Collect the precipitated soluble specific substance by centrifugation, redissolve in water, and repeat the procedure from the first addition of alcohol until all material precipitable with three-quarters of its volume of alcohol has been removed. In the case of the more soluble type-specific carbohydrates, material precipitable with as much as one volume of alcohol may be discarded by this fractionation. Redissolve the soluble specific substance in water and reprecipitate fractionally with alcohol until no change occurs in the precipitation reaction with homologous and heterologous immune sera, or until the total solids on an ash-free basis show the same percentage of Kjeldahl nitrogen. If the aqueous solution of soluble specific substance is not clear after centrifugation, filter through a final Mandler candle. After the last alcoholic precipitation, which is made from a clear solution, break up the soluble specific substance in several changes of alcohol and, finally, collect on hard paper in a Büchner funnel and wash repeatedly with absolute alcohol. Then grind in a mortar to a fine white powder and bring to constant weight in a vacuum desiccator over phosphorus pentoxide. Analyze for moisture, ash, Kjeldahl nitrogen, amino nitrogen, phosphorus, and acetyl. In a 1-per-cent solution, the biuret and iodine tests for protein and glycogen should be negative. Store in the dark in tightly stoppered amber bottles.

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CHAPTER 49

PRODUCTION AND STANDARDIZATION OF DIPHTHERIA ANTITOXIN

Diphtheria antitoxic serum for prophylactic and therapeutic use is produced at the present time by the immunization of horses against the detoxified toxin (toxoid) of *Corynebacterium diphtheriae*. The potency of the serum and of the concentrated and purified antitoxin is determined by its neutralizing action in guinea pigs when combined with standard diphtheria toxin. The flocculation test is also used to estimate the potency.

Previously, diphtheria toxin was used for immunization (1). More recently toxoid to which calcium chloride is added has proved to be a very satisfactory antigen and has induced a more rapid response.

Different strains of *C. diphtheriae* vary considerably in their virulence and toxigenic activities. This has been especially noted with strain No. 3203, isolated in this laboratory. The toxin of this strain appears to induce more severe constitutional reactions in horses than those of the Park-Williams No. 8 culture. Hence toxoid has particular advantages when this strain is used as antigen.

PRODUCTION OF ANTITOXIC SERUM

IMMUNIZATION OF HORSES

The immunization of horses is commenced by subcutaneous injections of a series of gradually increasing doses of diphtheria toxoid. Later, toxoid to which calcium chloride ($CaCl_2$) has been added is substituted for the fluid material.

Horses whose blood sera contain one-twentieth of a unit or more of natural diphtheria antitoxic substances per milliliter are selected when available. Since the response to immunization is relatively rapid, horses may be used which, on account of their age, are not considered desirable for prolonged periods of immunization.

Toxoid Used in Immunization.—Diphtheria toxoid with a flocculation value (Lf) of preferably not less than 20 is used for immunization. For preparation, see p. 734.

Injection and Dosage.—The general scheme for the first 23 injections of toxoid is given in table 73. All injections are made subcutaneously. After the second dose of 200 ml. has been given, each injection is usually increased by 25 ml. and injections are made at three-day intervals. The maximum dose of toxoid is 400 ml.

When toxin is used for immunization, gradually increasing doses are given subcutaneously beginning with 20 M.L.D. After a dose of 100,000 M.L.D. is reached, each injection is increased by 25 ml. and the interval to four days. The maximum dose of toxin is usually from 500 to 600 ml.

Temperature Reactions.—Temperatures are taken every morning. By following closely the general condition and febrile reactions, unfavorable results from injections and early phases of suppurative or other processes may be noted. Frequently, variations in regular schedules are advisable. If the temperature is above normal, the injection or the bleeding is postponed. When a bleeding is postponed for several days, one or more additional injections are usually given before bleeding.

TABLE 73

General scheme for injections of diphtheria toxoid

INJECTION NUMBER	DAY OF INJECTION	VOLUME OF TOXOID	REMARKS
		ml.	
1	1 (Thurs.)	5	Doses of less than 20 ml. are made up to that volume with salt solution.
2	2	5	
3	5	10	
4	6	10	Injections are made subcutaneously.
5	8	10	
6	9	20	Beginning with the 17th injection, sufficient 20-per-cent $CaCl_2$ solution to give a final concentration of 0.3 per cent is added to the toxoid at the time of injection.
7	12	20	
8	13	20	
9	14	40	
10	15	40	
11	16	60	
12	19	60	Trial bleeding.
13	20	80	
14	21	80	
15	22	80	
16	23	100	
17	26	100	Trial bleeding.
18	29	100	
19	33	150	Trial bleeding.
20	35	150	
21	37	150	
22	40	200	
23	43	200	

Twenty-per-cent $CaCl_2$ solution is prepared in a Pyrex flask, filtered through paper, and sterilized at 15 pounds pressure for thirty minutes.

Bleeding.—Trial bleedings are taken at least once each month and at the time of each large bleeding. They are usually taken on the third or fourth day after an injection.

Bleedings for plasma are taken when the titer of the serum reaches from 600 to 700 antitoxic units per milliliter unless, as frequently occurs, the tests indicate that a further rapid rise in titer may be expected. In this case the bleeding is delayed, usually until the titer appears to have reached its upper limit. Bleedings are taken seven days after an immunizing dose and injections are resumed on the following day. Between bleedings, four injections of toxoid at three-day intervals are usually given. Bleedings are generally continued until the antitoxic content of the serum falls to 500 units per milliliter or until the condition of the horse makes further immunization inadvisable. Treatment is then discontinued after a series of 2 or 3 bleedings or the horse is bled out, generally on the day following a bleeding. For technic of bleeding for plasma, see "Care and Use of Large Animals," p. 66.

TREATMENT OF PLASMA AND CONCENTRATED ANTITOXIN

Concentration and Purification.—Antitoxic plasma is concentrated and purified by the method given in "Concentration of Plasmas and Sera of Immunized Horses," p. 713. A 10-ml. sample is taken from the pooled material before and after concentration for potency tests.

Filtration of Antitoxin.—For filtration of antitoxin, see "Filtration of Prophylactic, Therapeutic, and Other Preparations," p. 806. After filtration, samples for tests of sterility and potency are taken. If contaminated, the antitoxin is refiltered as soon as possible.

Antitoxin for prophylactic use is diluted before filtration to contain 600 units per ml. The diluent is 0.8-per-cent salt solution containing 0.3 per cent of cresol.

STANDARDIZATION OF ANTITOXIN

Diphtheria antitoxin is standardized by determining its neutralizing action against a standard diphtheria toxin in comparison with the neutralizing action of a standard control antitoxin. The potency of the antitoxin is expressed in units per milliliter.

One unit of antitoxin is defined as the least amount of serum necessary to protect a 250-gram guinea pig for ninety-six hours against the test, or L+, dose of the standard toxin against which one unit of the control antitoxin protects similarly.

Three methods of testing are used. In the subcutaneous (2), or official, test the potency is measured by the degree of reaction induced by subcutaneous injections into guinea pigs of mixtures containing the antitoxin to be tested and the standard toxin, in comparison with the reaction induced by a similar mixture containing standard antitoxin. In the intracutaneous test (3–5) the degree of neutralization is measured by the local reactions induced when toxin-antitoxin mixtures are injected into guinea pigs. The flocculation test (6–11) is based on the assumption that flocculation takes place in a neutral mixture of toxin and antitoxin before it occurs in a mixture that contains an excess of either.

The subcutaneous method is used for the final test of all lots of antitoxin distributed for prophylactic or therapeutic use and as a control on the accuracy of the flocculation method. The intracutaneous method is used at present only to determine the natural diphtheria antitoxic content of the sera of normal horses and small amounts of diphtheria antitoxin in human sera. The flocculation method is used for tests of trial bleedings and preliminary tests of antitoxins.

SUBCUTANEOUS TEST

Different amounts of each antitoxin are mixed with the test, or L+, dose of standard toxin and injected subcutaneously into a series of guinea pigs. At the same time, as a basis for comparison, a mixture containing one unit of standard antitoxin and the test dose of toxin is tested in two guinea pigs.

Standard Antitoxin.—Standard diphtheria antitoxin used in determining the L+ dose of the toxin and as a control in all tests of antitoxic material is obtained in a glycerolated solution from the National Institute of Health. On each bottle are entered the dilution required to give one unit per milliiter and the expiration date.

Standard Toxin.—The toxin is standardized by determining its L+ dose against the Federal standard antitoxin. Since it is essential that the L+ dose of a standard toxin be accurately determined for the standardization of antitoxin, it is usually necessary to perform a large number of tests in selecting a new standard toxin. For preparation and preliminary standardization, see p. 624. The L+ dose of the toxin is determined for each new bottle of the same lot when first opened, and later if variations make it advisable. Control tests with standard antitoxin made in standardization tests of the antitoxins serve as a further test of the potency and stability of the toxin. When the control animals die somewhat too soon or too late in several consecutive tests, the L+ dose may usually be changed without restandardization of the toxin.

The L+ dose is defined as the least quantity of toxin which when added to one unit of antitoxin and injected subcutaneously will kill a guinea pig weighing 250 grams within ninety-six hours. In practice, however, the dose that kills in from seventy to eighty-five hours is selected. Since in standardization tests all the control guinea pigs injected with one L+ dose of toxin and one unit of standard antitoxin should die within ninety-six hours, allowance should be made for variations in susceptibility among the individual animals.

In determining the L+ dose of a standard toxin, mixtures of one unit of standard antitoxin and different doses of toxin—the range of doses selected at close intervals from the results of preliminary tests—not greater than

0.01 ml., occasionally at intervals of 0.003 or 0.002 ml., are tested subcutaneously in guinea pigs. For example, a series of doses might be 0.16, 0.15, and 0.14 ml. or 0.150, 0.147, and 0.145 ml. In final tests, three or four guinea pigs weighing between 240 and 260 grams are used to test each dose.

The toxin selected as standard is dispensed in 500-ml. bottles. It is covered with a thin layer of albolene, protected from light, and disturbed as little as possible. Samples required for tests are withdrawn in the cold room under special precautions.

The Test

Healthy guinea pigs weighing between 230 and 280 grams are used. Animals of not less than 250 grams and preferably not more than 260 grams are selected for the controls.

Dilutions.—The technic is that given for the standardization of diphtheria toxin, p. 626. The "to contain" pipettes must, however, be rinsed at least three times and the dilutions thoroughly mixed, since serum and antitoxin are more viscous than toxin.

Antitoxin: Depending upon how close an estimation of the potency is desired, from 3 to 6 doses of each antitoxin are tested. Dilute the antitoxin so that the desired amounts of antitoxin are contained in 1 ml.

Thus, if an antitoxin is assumed to contain from 2200 to 2400 units per ml., make 2200-, 2300-, and 2400-fold dilutions, and use 1 ml. for the dose.

Make the first dilution of all the samples, which should have reached room temperature, and then complete the dilutions of the first sample before proceeding to the second. Continue with the other samples in order. When all dilutions are completed, pipette 1 ml. of the final dilutions into small brown bottles with a 1- or 2-ml. certified graduated pipette, and arrange the bottles in sequence. With dilutions of the same sample, transfer the greatest dilution first, then wipe the tip of the pipette and draw up the next lower dilution and discard twice to rinse. Rinse between dilutions of different samples with salt solution twice and the dilution twice. After all the dilutions have been pipetted, add 1 ml. of salt solution to each bottle.

Use the same technic for the standard antitoxin. With a "to contain" pipette add 1 or 0.5 ml. to the required volume of salt solution, rinsing the pipette at least three times on account of the glycerol present.

Toxin: Pipette carefully from the stock bottle into a sterile tube sufficient standard toxin for the day's test. Dilute the toxin so that 1 ml. of the dilution contains the test dose (one L+ dose).

Toxin-Antitoxin Mixture.—With a certified 1- or 2-ml. graduated pipette, add to the antitoxin dilution in each of the bottles 1 ml. of the toxin dilution. Take care that the pipette does not come in contact with the antitoxin when touching the tip to the side of the container. Wipe the tip carefully after each drawing up and each emptying. Immediately after adding the toxin, rotate the bottle gently to mix the contents thoroughly, taking care that none of the mixture touches the cork. Allow about a minute between the addition of the toxin to each bottle in order to approximate the interval between injections, not less than thirty minutes and not more than one hour between adding the toxin to the antitoxin and the injection.

Injections. Observation of Animals. Necropsies.—The procedures are those given for the standardization of diphtheria toxin, p. 627.

Estimation of Potency.—A conservative estimate of the potency should always be made. The control guinea pigs should die in from sixty-five to eighty-five hours; the animals on which the titer of the material tested is based should survive ninety-six hours or longer. The dose of test antitoxin that protects under these conditions is considered to contain one unit of antitoxin.

In making the estimate, every factor must be considered, such as the original weight and condition of the guinea pig, signs of illness, relative reactions of different animals of a series, autopsy findings, and particularly the condition and time of death of the two guinea pigs that received the control antitoxin. If the results of the test are indeterminate because a suitable range of dilutions was not selected, or because the animals died irregularly, the test should be repeated.

INTRACUTANEOUS TEST

In testing antitoxin by this method, the reactions induced in guinea pigs by intracutaneous injections of mixtures containing a test dose of standard diphtheria toxin and varying dilutions of antitoxin are compared with that induced by a mixture containing the same dose of toxin and 1/10,000 of a unit of standard control antitoxin injected at the same time on the same animal. Generally one guinea pig is used for each test.

This method of testing is always used to determine the natural diphtheria antitoxic substances in normal sera. Tests for 1/500, 1/100, and 1/20 of a unit per milliliter are made.

Standard Antitoxin.—The standard antitoxin is the same as that used in the subcutaneous test.

Standard Toxin.—The standard toxin is the same as that used in the subcutaneous test. To determine the intracutaneous test dose, prepare dilutions of the toxin differing by approximately 10 per cent above and below one containing 1/500 of an L+ dose per milliliter. Mix 1 ml. of each dilution with 1 ml. of the dilution of standard antitoxin containing 1/500 of a unit per milliliter, and inject 0.1 ml. of the mixture intracutaneously into a guinea pig. The doses injected contain 1/10,000 of an L+ dose of toxin, or slightly more or less, and 1/10,000 of a unit of standard antitoxin. The amount of toxin in the mixture inducing a slight but definite redness in twenty-four hours beginning to fade after forty-eight hours is considered the test dose of toxin. At least three guinea pigs are used for the test so that slight variations in the individual animals may be considered in making the final estimates.

The Test

White guinea pigs weighing between 350 and 450 grams are used. For preparation of the animals for injection, see detailed directions, p. 77.

Dilutions.—The dilutions are made in salt solution according to the procedures described for "Production and Standardization of Streptococcus Toxin," p. 643.

Antitoxin: Dilute the test and standard control sera so that the desired amounts of serum are contained in 0.05 ml.

For the standard control antitoxin, prepare a dilution that contains 1/500 of a unit per ml. In tests for 1/500 or 1/20 of an antitoxic unit per milliliter, use the undiluted material for the first, and for the second, prepare a 25-fold dilution.

Toxin: Make a dilution of the standard toxin that contains 20 test doses per ml., i.e., one test dose in 0.05 ml.

Toxin-Antitoxin Mixtures.—Follow the procedure described on p. 672. Prepare the mixtures in brown glass bottles instead of tubes. Pipette 1 ml. of each dilution of antitoxin to be tested into a series of bottles and add 1 ml. of the standard-toxin dilution. Mix the contents thoroughly by a rotary motion. Allow the mixtures to stand at room temperature for from 30 to 60 minutes before injecting.

Injections.—Six or seven injections, at least one inch apart, may be made on each side of a guinea pig, depending upon the size of the animal and the condition of the skin. The standard-antitoxin dilution is usually injected in the center on one side. Inject 0.1 ml. of each of the toxin-antitoxin mixtures, beginning each series with the mixture containing the highest dilution of serum. Before injecting any dilution, rinse the syringe twice with it; between different sera, first rinse the syringe twice with salt solution.

Readings and Estimation of Potency.—Make readings at the end of twenty-four, forty-eight, and seventy-two hours, and record the degree of redness, and also the induration, necrosis, scaling, or pigmentation.

Estimation of the potency is based on the relative degree of reactions induced in forty-eight hours. The standard-antitoxin control should show a slight but definite redness which begins to fade after forty-eight hours. Since slight variations in the reactions may occur in different guinea pigs, in interpreting the reactions on any one animal, the control reaction on that animal should be used as the basis for comparison.

FLOCCULATION TEST

Different amounts of antitoxin are mixed with a constant amount of a standard toxin, and the mixtures are incubated at 40°C. until flocculation appears in one of the tubes.

Standard Toxin.—The toxin selected is standardized by determining its flocculation value (Lf) against the standard serum, see p. 628. Toxin prepared in infusion-free peptone medium (1), that has a titer not lower than 10 and that gives regular, well-defined flocculation with the standard serum is selected. It is tested at approximately half-unit intervals. A bottle of the toxin selected is removed from the cold room when needed and fitted with a siphon and a 100-ml. burette with a silver tip, 7 cm. long with bore 1.5 to 2 mm., protected with a glass bell. It is kept at room temperature and out of direct sunlight while in use, usually not longer than five weeks. Satisfactory results have, however, been obtained with toxin that has stood at room temperature for from two to three months. The toxin in each bottle is tested against the standard serum before it is used as a standard.

The Test

Measure with 1.0- or 0.2-ml. pipettes the selected amounts of the test antitoxin into a series of tubes (133 by 16 mm., of uniform diameter). Usually a test consists of a series of five tubes. To each add 10 ml. of the standard toxin from the burette. Stopper the tubes and mix with a single, quick, up-and-down motion. Incubate in a constant-level water bath held at 40°C., with the water adjusted so that the liquid in the tubes is immersed to about two-thirds of its height.

Readings and Estimation of Titer.—Readings are made by artificial light against a dark background at appropriate intervals to observe initial flocculation. The estimation of the titer is based on the amount of serum with which the initial reaction occurs. If flocculation occurs simultaneously in two tubes, an approximate estimate may be made; if in more than two or in the first or last of the series, the test must be repeated.

The titer is estimated as follows:

$$\text{No. of units} = \frac{\text{Lf value} \times \text{ml. standard toxin}}{\text{ml. antitoxin}}$$

PREPARATION FOR DISTRIBUTION

After the sterility tests have been satisfactorily completed and the titer has been determined, the antitoxin is dispensed in 1000-unit doses for prophylactic use, and in 5000- and 10,000-unit doses for therapeutic use, with an excess of 20 per cent. Diphtheria antitoxin is considered satisfactory for use, if kept under proper conditions, for eighteen months from the date of the last satisfactory test.

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CHAPTER 50

PRODUCTION AND STANDARDIZATION OF TETANUS ANTITOXIN

Tetanus antitoxic serum is produced for prophylactic and therapeutic purposes by the immunization of horses against the toxin of *Clostridium tetani*. The potency of the serum and of the concentrated and purified product is determined by its neutralizing action in guinea pigs when combined with standard tetanus toxin. The procedures for the production of the antitoxin correspond closely to those described for diphtheria antitoxin.

The substitution of toxoid for toxin has been recommended and its value during the early stages of immunization has been established (1, 2). In our experience, serum of high titer was not obtained with toxoid or with toxoid precipitated with calcium chloride or aluminum potassium sulfate. Toxin precipitated with alum, however, has proved an effective antigen (3).

PRODUCTION OF ANTITOXIC SERUM

IMMUNIZATION OF HORSES

Relatively young horses that have received at least two routine immunizing injections of tetanus toxoid, the second preferably six or more months previously, are usually selected.

Toxin Used in Immunization.—Tetanus toxin containing from 10,000 to 20,000 or more minimum lethal doses per milliliter is used for immunization, see p. 632.

Injection and Dosage.—An initial subcutaneous injection of 20 ml. of tetanus toxoid to which has been added sufficient 20-per-cent aluminum potassium sulfate solution to give a 0.5-per-cent concentration is given irrespective of the interval since the previous immunizing dose of toxoid. One month later injections of toxin at three-day intervals are commenced, the number of M.L.D. in the first three doses depending upon the antitoxic content of the serum.

The general scheme for initial injections and doses is given in table 74. Occasionally modifications are required. All injections are made subcutaneously. After a dose of 100,000 M.L.D. of toxin, the horse is rested for one month. On reimmunization, aluminum potassium sulfate is given with the toxin. After a dose of 75 ml. has been reached, each injection is generally increased by 25 ml. When aluminum potassium sulfate is added to the toxin the maximum dose is 200 ml. and injections are made at 4-day intervals. Between bleedings 4 to 5 injections at 4-day intervals are usually given.

Twenty-per-cent aluminum potassium sulfate solution is prepared by sterilizing the crystals in hot air in a Pyrex flask and then adding hot sterile distilled water immediately.

TABLE 74

General scheme for injections of tetanus toxin

INJECTION NUMBER	M.L.D.	VOLUME		REMARKS
		ml.		
1	5,000	0.5*		About one month before the initial dose of toxin, an injection of tetanus toxoid with aluminum potassium sulfate is given.
2	10,000	1.0*		
3	20,000	2.0*		
4	25,000	2.5		
5	37,500	3.7		
6	50,000	5.0		Doses of less than 20 ml. are made up to that volume with salt solution.
7	70,000	7.0		
8	100,000	10.0		
	Rest one month			Injections made subcutaneously every third day.
9	5,000	0.5		
10	15,000	1.5		Toxin contains 10,000 M.L.D. per milliliter.
11	35,000	3.5	plus aluminum potassium sulfate	
12	55,000	5.5		Beginning with ninth injection, sufficient 20-per-cent aluminum potassium sulfate solution is added to the toxin at the time of injection to give an approximate final concentration of 0.25 or 0.5 per cent.
13	75,000	7.5		
14	105,000	10.5		
15	135,000	13.5		
16	175,000	17.5		
17	215,000	21.5		
18	265,000	26.5		
19	330,000	33.0		
20	385,000	38.5		
21	450,000	45.0		
22	550,000	55.0		
23	750,000	75.0		

* Dosage of toxin in the first three injections may vary depending upon the titer of the serum following preliminary immunization.

Bleedings.—Bleedings for plasma are taken when the serum reaches from 700 to 800 antitoxic units per milliliter. They are taken on the seventh day after a dose of toxin and injections are resumed on the following day. When the titer has dropped below 500 units, immunization is usually discontinued. It may be advisable to rest a horse producing potent antitoxin for two or three months at intervals of one or two years.

TREATMENT OF PLASMA AND CONCENTRATED ANTITOXIN

Concentration and Purification. Filtration.—For procedures see "Production and Standardization of Diphtheria Antitoxin," p. 653.

Antitoxin for prophylactic use is diluted before filtration to contain 900 units per ml. The diluent is 0.8-per-cent salt solution, containing 0.3 per cent of cresol

STANDARDIZATION OF ANTITOXIN (4–6)

Tetanus antitoxin is standardized by determining its neutralizing action against a standard tetanus toxin in guinea pigs in comparison with the neutralizing action of a standard control antitoxin. The potency of the antitoxin is expressed in units per milliliter.

One unit of antitoxin is defined as "ten times the least amount of serum necessary to save the life of a 350-gram guinea pig for ninety-six hours against the official test dose of the standard toxin," (4) against which one-tenth of a unit of the control antitoxin protects similarly. This unit value is twice that of the International unit defined by the Health Organization of the League of Nations (6).

SUBCUTANEOUS TEST

Different amounts of each antitoxin are mixed with the test dose of standard toxin and injected subcutaneously into a series of guinea pigs. As a basis of comparison in estimating the potency of the antitoxin, a mixture of one-tenth of a unit of the control antitoxin and the same test dose of toxin is tested at the same time in two guinea pigs.

Standard Antitoxin.—The standard antitoxin is obtained in a glycerolated solution from the National Institute of Health. The dilution required to give one-tenth of a unit per milliliter is given on the label.

Standard Toxin.—The standard tetanus toxin is supplied in powder form in a sealed tube by the National Institute of Health. The identifying letter and the amount required for the test dose are recorded on the label. If kept under proper conditions, the toxin may be used for a number of months. The extent of deterioration is determined by control tests against standard antitoxin and the test dose is changed as required.

To preserve the toxin under favorable conditions, prepare a desiccator with fresh, concentrated sulfuric acid and seal with vaseline. Record the date on the label of the new tube, open, and place it in the desiccator. Cover the latter and store in the cold room where it will not be disturbed. Do not allow cotton or other substances likely to retain moisture to remain in the desiccator. Replace the sulfuric acid after every fifth opening.

Since tetanus toxin is one of the most powerful poisons known, caution should be exercised in opening a new tube and in handling the dried or diluted material Typical symptoms of tetanus from inhalation of the powder have been reported.

The test, or L+, dose of the standard toxin is verified by testing subcutaneously mixtures containing one-tenth of a unit of antitoxin and a series of slightly different doses (intervals of 0.00002 or 0.00004 gram) of the toxin, the median of which is the dose indicated on the label. Two guinea pigs are used for each mixture. For procedure and determination of L+ dose, see p. 633. The test dose selected is the one that kills in from sixty-five to eighty-five hours.

The Test

Guinea pigs weighing between 330 and 380 grams are used. Animals of not less than 350 grams, and preferably not more than 360, are selected for the controls.

Dilutions.—Antitoxin: For procedure, see "Production and Standardization of Diphtheria Toxin," p. 626. The doses tested are in the range of one-tenth of a unit of antitoxin.

Toxin: To prevent absorption of moisture from the air, weigh accurately and as quickly as possible an amount of toxin judged to be sufficient for the day's work. Handle the tube and the flask in which the material is weighed with a piece of clean cheesecloth. Dissolve the toxin in an amount of salt solution such that the test dose will be contained in 1 ml. The number of milliliters of salt solution required is equal to the weight of toxin taken divided by the weight of the toxin in the test dose.

Assuming the weight of the toxin taken to be 0.0182 gram and the test dose to be 0.0008 gram, then the volume of salt solution required will be 22.5 ml., that is, $\frac{0.0182}{0.0008}$.

Use the toxin dilution within one hour after it is made.

Toxin-Antitoxin Mixture.—For procedure, see "Production and Standardization of Diphtheria Antitoxin," p. 655.

In testing for small amounts of antitoxin, as in serum from persons immunized against tetanus toxoid or from horses that have had preliminary immunization with the toxoid, if the potency of the serum is 1/20 of a unit or more, varying amounts of the serum are combined with the test dose of standard toxin. If the antitoxic content of the serum is less, fractions of the test dose are used. The reactions induced by these mixtures when injected subcutaneously are compared with those induced by similar mixtures that contain standard antitoxin. Preliminary tests for 1/100, 1/10, and 1/2 of a unit per milliliter are usually made.

Injections. Observation of Animals. Necropsies.—For procedures, see "Production and Standardization of Tetanus Toxin," p. 634.

Estimation of Potency.—A conservative estimate of the potency should always be made. The control guinea pigs should die in from sixty-five to eighty-five hours; the animals on which the titer of the material tested is based should survive ninety-six hours or longer. The dose of test antitoxin that protects under these conditions is considered to contain one-tenth of a unit of antitoxin.

PREPARATION FOR DISTRIBUTION

The antitoxin is dispensed in doses of 1500 units for prophylactic use and 20,000 units for therapeutic purposes with an excess of 20 per cent. If kept under proper conditions, the material is considered satisfactory for use for eighteen months from the date of the last satisfactory test.

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CHAPTER 51

PRODUCTION AND STANDARDIZATION OF BOTULINUS ANTITOXIC SERA

A small supply of univalent botulinus antitoxic serum of type A and of type B is prepared for therapeutic and diagnostic purposes. The sera are produced by the immunization of horses against the homologous toxins of *Clostridium botulinum.* The potency of the sera is determined by their neutralizing action in guinea pigs when combined with their respective toxins.

Production of univalent antitoxic serum against the toxin of *Cl. botulinum* type E has not seemed warranted. Type-E strains have proved to be the incitants of food poisoning in New York State in only two instances (1, 2). Types C and D have rarely been associated with botulism in man.

PRODUCTION OF ANTITOXIC SERA

IMMUNIZATION OF HORSES

The method corresponds to that given for the production of diphtheria antitoxic serum. The initial injection of toxin is very small and, until antitoxin has been demonstrated in the blood, increase in dosage is gradual since the lethal dose for a horse approximates 100 M.L.D.

If severe reactions occur when horses are being immunized against *Cl. botulinum* type B by the method indicated in table 75, botulinus toxoid may be used. Satisfactory results have been obtained when gradually increasing doses of calcium-precipitated toxoid were given at 14-day intervals for from six to eight months, followed by toxin with 0.25 per cent of aluminum potassium sulfate.

Toxins Used in Immunization.—Satisfactory results have been obtained by using toxins containing from 1000 to 2000 minimum lethal doses per milliliter during the early stages of immunization and substituting a more potent toxin later.

Injection and Dosage.—The general scheme for injections is given in table 75. When antitoxin is demonstrated in the serum, the doses of toxin are increased from 40 to 50 per cent up to 100 ml.; then increases of from 30 to 50 ml. are made up to 500 ml., the maximum dose given.

If a more potent toxin is to be substituted, the dose is reduced proportionately. When the dose is less than 1 ml. of toxin, a dilution in salt solution containing one dose per milliliter is made on the day of injection. At the time of injection 1 ml. of this dilution is diluted further to 20 ml.

Bleedings. Trial bleedings are taken immediately before the 14th injection, then once each month or more often if advisable. Large bleedings may be taken when the titer of the serum is adequate. The maximum titer of type-A serum as yet obtained has been 2000 units, of type-B, 600 units. If the material is to be concentrated, bleedings are taken for plasma.

TABLE 75

General scheme (3) of injections of botulinus toxin up to 1000 M.L.D.

INJECTION NUMBER	NUMBER OF M.L.D.	VOLUME	REMARKS
		ml.	
1	1/20	0.00005	Doses of less than 20 ml. are diluted to that volume with salt solution. Injections subcutaneously every third or fourth day. Toxin contains 1000 M.L.D. per ml. When preliminary injections of botulinus toxoid have been given, the initial doses of toxin are based on the unit content of the horse's serum.
2	1/10	0.0001	
3	1/5	0.0002	
4	1/2	0.0005	
5	1	0.001	
6	2	0.002	
7	4	0.004	
8	6	0.006	
9	8	0.008	
10	12	0.012	
11	16	0.016	
12	24	0.024	
13	32	0.032	
14	40	0.04	
15	50	0.05	
16	60	0.06	
17	80	0.08	
18	100	0.10	
19	130	0.13	
20	160	0.16	
21	190	0.19	
22	240	0.24	
23	290	0.29	
24	340	0.34	
25	390	0.39	
26	470	0.47	
27	550	0.55	
28	650	0.65	
29	1,000	1.0	

TREATMENT OF SERA AND PLASMA

For the treatment of the sera, see that of antimeningococcus serum, p. 700; for the concentration of the plasma, see p. 713.

STANDARDIZATION OF ANTITOXIC SERA

Botulinus antitoxic sera are standardized by determining their neutralizing action when combined with the homologous standard toxins. Toxins at least two years old are selected and are standardized against the standard control sera supplied by the National Institute of Health (4).

The test, or L+, dose of toxin contains approximately one hundred M.L.D. or the amount of toxin which when mixed with one-tenth of a unit of the homologous standard antitoxin and injected subcutaneously will kill a 250-gram guinea pig in about ninety-six hours. The L+ dose is determined according to the method for diphtheria toxin (see p. 628) except that one-tenth of a unit of antitoxin is used. Guinea pigs weighing between 230 and 280 grams are selected. The procedure of injection, the symptoms after injection, and the lesions encountered at autopsy are the same as those described for botulinus toxins.

PREPARATION FOR DISTRIBUTION

Botulinus antitoxic sera are dispensed in 20-ml. doses for therapeutic use and in 5-ml. amounts for diagnostic purposes. If kept under proper conditions, they are considered satisfactory for use for eighteen months from the date of the last satisfactory test.

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CHAPTER 52

PRODUCTION AND STANDARDIZATION OF ANTISTREPTOCOCCUS SERUM

Antistreptococcus serum for the treatment of scarlet fever, erysipelas, and other streptococcus infections is produced by the immunization of horses against live culture and toxin of representative strains of hemolytic streptococci. The serum is concentrated and purified. The potency of the serum is determined by its neutralizing action in rabbits and persons when combined with standard streptococcus toxin.

The results of studies in this laboratory over a period of years of the toxigenic properties of approximately 2000 strains of hemolytic streptococci from human infections, have shown that antistreptococcus serum produced with the standard strain No. 165 (Dochez N. Y. 5), neutralizes approximately 75 per cent of all the toxins irrespective of the clinical manifestation (1, 2). This strain has proved to be a broadly valent and highly potent antigen (3, 4). By toxin-antitoxin neutralization tests, it has been possible to divide the large number of strains studied into several toxin groups. No evidence has been obtained, however, to indicate the specificity of any of these groups for a particular form of infection (2). The therapeutic value of the serum in different streptococcus infections has been definitely established (5, 6).

The results of further investigation indicate that a multivalent serum produced with two strains of different toxin groups, Nos. 165 and 32369, neutralizes the toxins of all the representative strains of the various toxin groups that have been studied (7, 8). On the basis of these results, serum for the treatment of streptococcus infections is now produced with both strains.

Strains

The standard strains, Nos. 165 and 32369, are used for the production of therapeutic antistreptococcus serum. See "Production and Standardization of Streptococcus Toxin," p. 639.

PRODUCTION OF SERUM

IMMUNIZATION OF HORSES

The immunization of horses is commenced by the "agar culture" method (9). Later the injection of live streptococci is supplemented by the subcutaneous injection of the toxic filtrate of broth cultures. Horses with discharging abscesses are isolated so far as practicable. A rest period of from one to three months each year, or more frequently if indicated by the condition of the horse, is advisable. The production period may extend for six years or longer.

At present, the multivalent serum is prepared by pooling the concentrated sera produced against each of the two strains. Experience has shown that a more favorable response in horses is obtained when one strain instead of two is used as antigen.

Preparation of Culture and Toxin.—Culture: Use the second broth transfer from the latest heart-blood broth culture that has been tested for purity. For the first transfer, pipette 0.1 ml. of the heart-blood culture into a tube of beef-infusion broth (*F5A*), streak a blood-agar plate (*F43A*), and incubate at approximately 35°C. for from twelve to sixteen hours. Examine the plate culture and a slide preparation of the broth culture stained by Gram's method. If no contamination is indicated, pipette from 0.5 to 1 ml., depending upon the volume, into the required amount of broth, streak a blood-agar plate, and incubate the cultures at approximately 35°C. for from twelve to sixteen hours. Examine as previously described. If pure, centrifuge the broth culture until the supernatant is clear, draw it off and discard. Suspend the cells in 2 or 3 ml. of broth. Rinse the centrifuge bottles with 1 or 2 ml. of broth and add the washings to the suspension. The final volume should not be more than 5 ml. Streak a blood-agar plate with a small loopful of the suspension.

Toxin: Use the toxic filtrate from a 7-day broth culture, see p. 640. The standard toxins contain from 30,000 to 50,000 skin test doses (S.T.D.) per milliliter and are usually not more than three or four months old.

No definite period has been determined after which toxin is not satisfactory. A relatively fresh product has always been used.

Toxins of from 100,000 to 200,000 S.T.D. per milliliter have also been used for the immunization of horses, see p. 640. Although from two and one-half to five times as much toxin was given, since the volume injected equalled that of toxins of lower titer, the potency of the serum was not increased. Toxins concentrated to equal twenty times the original titer, according to the acetone-precipitation method (10), and given in maximum doses of 300 ml. failed to increase the potency. In some horses the titer of the serum was increased when injections of toxin to which potassium alum or calcium chloride had been added were substituted for the fluid toxin.

Injection and Dosage.—A series of 3 or 4 injections of live microorganisms is given first. The interval between the injections, usually from two to four weeks, depends upon the extent and severity of the abscess or abscesses induced. First 40 ml. of 1.75-per-cent beef-infusion agar (*F33*) are injected subcutaneously in the saddle region and allowed to harden for a few minutes after which the culture is inoculated into the agar. See "Care and Use of Large Animals," p. 64. The first dose consists of the cells from 25 ml. of broth culture; the second, usually those from 50 ml. Succeeding doses are either increased by the addition each time of streptococci from 50 to 100 ml. of broth culture or the same dose is repeated, depending upon the reaction induced. The maximum dose contains microorganisms from 300 ml. of broth culture.

The first injection of toxin, which is given subcutaneously at 3-day intervals, is made from two to four months after immunization is commenced, and as soon as the abscess from the previous injection commences to subside. The initial dose is 10 ml., from 300,000 to 500,000 S.T.D.; the second, generally 25 ml. Subsequent doses are increased progressively by 25 or later even by 50 ml., until a total of 500 ml. is reached. At this time the interval is four instead of three days. The maximum dose of toxin to which potassium alum or calcium chloride has been added is 300 ml. When toxins of two strains are used, alternate injections of each are given at the usual interval.

Injections of live microorganisms are continued at from one- to two-month intervals after those of toxin have been commenced. They are usually given on the same day as the homologous toxin but on the opposite side of the horse.

Reactions.—Temperatures are taken each morning. The day following an injection of live culture, a slight to moderate febrile reaction (from 101° to 103°F.) may be noted. This is usually transient and subsides within from twenty-four to forty-eight hours. Occasionally during the early period of immunization, a local reaction manifested by subcutaneous edema which may extend to the thoracic and abdominal regions may develop. Injections of toxin are suspended until it has disappeared.

Bleedings.—Trial bleedings are taken before the first dose of culture is given and at suitable intervals during early immunization, not sooner than the third or fourth day after an injection of toxin and usually immediately preceding one of culture. At present, bleedings are taken for plasma when the serum contains from 400 to 500 units of antitoxin per milliliter. They are taken on the sixth day after an injection of toxin, provided there is no active abscess present. Injections are resumed on the following day; culture and toxin are usually given. If the potency of the serum is 800 units or more, the injection of culture may be omitted and from one to three doses of toxin given between two bleedings. In a few instances two bleedings have been taken in succession, four or more days apart, with no evidence of appreciable drop in titer.

Experience at this laboratory indicates that approximately one year of immunization is required before a horse produces serum containing from 200 to 300 units per milliliter. After two years, the titer of the serum in some horses may reach 500 or 600 units, after three years, 800 or more. The highest-titered serum thus far obtained with strain No. 165 contained 1500 units per milliliter; with strain No. 32369, 4000 units.

IMMUNIZATION OF GOATS

Sera are produced in goats against representative strains of hemolytic streptococci for use in neutralization tests of toxins on goats. Large goats that have been used previously for the standardization of toxins are usually selected. Subcutaneous injections of increasing doses of toxin are given at from three- to four-day intervals. The initial dose is approximately 20,000 S.T.D. of toxin diluted to 20 ml. Subsequent doses are increased by 1 or 2 ml., later by 5 or 10 ml. until a maximum dose of 100 ml. is reached. Bleedings for serum, from 300 to 700 ml., are taken when the potency of the serum equals 20 or more units per milliliter. Cresol to give a 0.3-per-cent concentration is added as preservative. The valency of antistreptococcus sera produced in goats with certain strains appears to be broader than that produced with the same strains in horses (11).

TREATMENT OF PLASMA AND SERA

Concentration and Purification. Final Filtration.—The plasma is concentrated and purified. A 10-ml. sample is taken from the pooled plasma before and after concentration for potency tests on rabbits. After these tests are completed, the concentrated material is diluted, if necessary, with 0.8-per-cent salt solution, containing 0.3 per cent of cresol, to give the desired potency, at present between 800 and 1000 units per milliliter.

When bleedings are taken for serum, if the material is not to be concentrated and purified, cresol to give a 0.3-per-cent concentration is added as preservative, see p. 700.

Since precipitation may continue even after several months, the material is not filtered until shortly before it is dispensed. Tests for sterility and potency are made on the filtered product. If contaminated, it is refiltered as soon as possible.

STANDARDIZATION OF SERUM

Antistreptococcus serum is standardized by determining its neutralizing action against a standard toxin in intracutaneous tests on rabbits and persons in comparison with the neutralizing action of a standard control serum. The potency of the serum is expressed in units per milliliter.

One unit of antitoxin is the amount of antitoxin that neutralizes 50 S.T.D. of standard toxin. The antitoxic potency of the antistreptococcus serum, unconcentrated and concentrated, distributed by this laboratory has averaged 800 units per milliliter.

Standard Control Serum.—The standard control serum is obtained from the National Institute of Health. It was produced with the Dochez N. Y. 5 strain, dried, and dissolved in glycerolated salt solution to a volume ten times the original. The preparation contains 40 units per milliliter. Univalent sera produced with representative strains of streptococci have been standardized in this laboratory for control sera; unconcentrated sera not less than four months old are selected.

Standard Toxin.—The standard toxin, strain No. 165 (Dochez N. Y. 5), is the same as that used in the standardization of streptococcus toxin. A stable potent toxin prepared with strain No. 32369 has also been selected as a standard for tests of sera produced against this strain. It contains 30,000 S.T.D. per milliliter.

TESTS ON RABBITS

Different amounts of each serum are mixed with the test dose of standard toxin and injected intracutaneously in a 0.1 ml. dose into rabbits. For selection of rabbits, see "Production and Standardization of Streptococcus Toxin," p. 643. Generally two rabbits are used for each test. As a basis of comparison in estimating the neutralizing potency of the sera, mixtures of different amounts of the control serum and the same test dose of toxin are injected. As a control of the reactivity of the animal, the test dose of toxin, heated and unheated, and the largest dose of each serum are injected. Five skin test doses of toxin are generally used as a test dose, occasionally 10.

Satisfactory tests have been obtained with 10 S.T.D. of toxin instead of 5 in the occasional rabbit which gives only a slight reaction to the toxin in the preliminary test.

The Test

Dilutions.—The dilutions are made in salt solution according to the procedures described for streptococcus toxin, see p. 643.

Serum: Depending upon how close an estimation of the potency is desired, from 3 to 6 doses of each serum are tested. The doses in succession should be between one-half and three-quarters of the preceding amount. Dilute the test and standard control sera so that the desired amounts of serum are contained in 0.05 ml.

Toxin: Dilute the standard toxin so that the test dose is contained in 0.05 ml.

Thus, if the mixture to be injected is to contain 0.00002 ml. of serum and 0.0001 ml. of toxin, prepare a 2500-fold dilution of the former and a 500-fold dilution of the latter.

Serum-Toxin Mixtures and Controls.—Beginning with the largest fold dilution of serum, mix each thoroughly in turn and pipette exactly 1 ml. into a tube; pipette 1 ml. of the smallest-fold dilution of each serum into each of 2 tubes. The same pipette is used for all sera and is rinsed twice between different dilutions with the next to be pipetted, and between different sera twice with salt solution and twice with the next dilution to be pipetted. With a different pipette, add exactly 1 ml. of the toxin dilution to each tube, except to one of the 2 tubes containing the smallest fold dilution of each serum. For this purpose use one pipette throughout the test. To prepare the serum controls, add 1 ml. of salt solution to the tube containing the smallest-fold dilution of each serum but no toxin. For the toxin and heated-toxin controls add 1 ml. of salt solution to each of 2 tubes containing 1 ml. of the toxin dilution only. Take care that no material for the heated-toxin control touches any part of the tube that will not be immersed in the bath. Mix the contents thoroughly by a rotary motion. Heat the toxin for the heated control in a boiling water bath for two hours. Allow the mixtures to stand at room temperature for at least one-half hour before injecting.

Injections.—The procedures are the same as those given for streptococcus toxin, p. 644, with the following exceptions. Use separate syringes for the serum, toxin, and heated-toxin controls; between different preparations rinse with salt solution and then with the next material to be injected. Use one syringe for the serum-toxin mixtures and with each serum rinse between different mixtures with the next to be injected; between different sera, rinse first with salt solution and then with the next mixture to be injected. The mixtures that are expected to give the end-point in the titration are injected in the center of each row. The first series of injections in a row is therefore commenced with the serum control and followed by the mixture that contains the least amount of serum. The second series in the same row is started with the mixture that contains the largest dose of serum and ends with the serum control. The mixtures of standard control serum and toxin are injected in the second row. Injections of the toxin and heated-toxin controls are usually made at the end of a row in which only one long or two short series of mixtures are injected. Usually three rows of from 10 to 12 injections can be made on each side of a rabbit.

Readings and Estimation of Potency.—Readings are made in a bright light from twenty to twenty-four and from forty-four to forty-eight hours after injection. Record the degree of redness and swelling induced and the size of the reaction (vertical and horizontal diameters) in millimeters. Any reaction measuring less than 10 mm. in both diameters is considered negative. The potency is generally estimated on the basis of the later reading; occasionally, when it is questionable, both are considered. If the later reading is unsatisfactory because the reactions have faded, as often happens in tests with toxins of strains other than No. 165, or because the skin is irritated, an approximation of the potency can be made from the earlier reading. No test is considered satisfactory unless at least one dose of the test serum and one dose of the standard serum neutralize the test dose of toxin, and unless the next smaller dose of each serum fails to neutralize. A definite reaction should be induced by the toxin, and no reactions by the sera and heated-toxin controls. In final tests of sera, the amount of serum in the unneutralized mixture should be two-thirds or three-quarters the amount contained in the neutralized mixture; for preliminary tests and those of trial bleedings, one-half will give an adequate approximation.

The potency of antistreptococcus serum is estimated by determining the relation of the neutralizing dose of test serum to that of the standard control serum and is based on similar results obtained in two satisfactory tests. Thus, if the smallest dose of the test serum that neutralizes the test dose of toxin is 0.00004 ml. and the smallest dose of the standard control serum is 0.001 ml., the estimated potency of the test serum, based on a unit value of 40 units for the standard control serum is 25 times that of the control serum, or 1000 units per milliliter.

TESTS ON PERSONS

In addition to the tests on rabbits, each lot of material prepared for therapeutic use is standardized similarly against the standard toxin of strain No. 165 on at least two persons in comparison with the standard control serum. Persons in whom 1 S.T.D. of the standard toxin induces reactions measuring between 20 by 20 mm. and 30 by 30 mm. are the most satisfactory. No reaction should be induced by either the heated-toxin control or 0.0002 ml. of normal horse serum. One skin test dose of toxin is used as a test dose. The same control injections are made as in the tests on rabbits.

Similar results were obtained in comparative titrations with test doses of 1 S.T.D. of toxin and of 5 (the Federal test dose). The smaller dose has proved very satisfactory and has the advantage of requiring smaller amounts of serum. In repeated tests on the same individual the possibility of either local immunization or sensitization of the skin with the serum-toxin mixtures is also lessened. Moreover, certain individuals who react to larger amounts of serum are quite satisfactory for tests with smaller doses.

The serum is not standardized on persons against the toxin of strain No. 32369. Although this toxin induces reactions in persons similar to those induced by the standard toxin, strain No. 165, when it is combined with serum, even in mixtures that are definitely not neutralized according to tests on rabbits, the capacity of the toxin to induce a reaction in the skin is apparently destroyed. Such reactions as do occur are very pale and slightly purple and resemble somewhat a faded typical reaction four or five days after the injection. Reactions induced in rabbits by mixtures of serum and toxin of strain No. 32369 from twenty to twenty-four hours after injection are similar to those induced by mixtures of serum and toxin of strain No. 165; within forty hours after injection, however, the former have frequently faded and the latter have increased in size and intensity.

The Test

From 4 to 6 doses of each serum are tested. The doses in succession should be two-thirds or three-quarters of the preceding amount. With aseptic precautions, prepare the dilutions, the serum-toxin mixtures, and the controls as for the tests on rabbits.

Because of the variation in susceptibility of different individuals, which cannot be gauged by their reaction to toxin, it is necessary to use from 4 to 6 doses of each serum to be tested to insure an end point in the titration. Variations in susceptibility although not great also occur in the same individual as shown by tests performed at different times.

Injections.—Use syringes and needles reserved for human tests and the same precautions as for tests of streptococcus toxins on persons. Provide the same number of syringes and rinse them between injections, as in tests of serum on rabbits. Commence a series of injections with the smallest dose of serum and place corresponding mixtures of test serum and toxin and of standard control serum and toxin in approximately the same relative positions. Avoid, if possible, injecting a series of mixtures in more than one row. Two rows of from 4 to 6 injections, 3 or 4 cm. apart, can be made conveniently on the flexor surface of each arm. Begin the injection of a series just below or not more than 12 cm. above the elbow and continue to within 7 cm. of the wrist. If more than 18 or 20 injections are to be included in a test, the anterior surface of the thigh is used. On each, 3 rows of from 4 to 6 injections about 4 cm. apart can be made. Begin each row on the upper thigh and continue to within from 8 to 10 cm. of the knee. Men are generally used for tests on the arm, women for tests on the thigh.

A period of three months is allowed between tests on the same individual. Some persons have been used for as many as 15 satisfactory tests; others, for only 1 or 2, after which the susceptibility was apparently permanently changed.

Readings and Estimation of Potency.—The procedures are the same as those for tests on rabbits.

PREPARATION FOR DISTRIBUTION

After the standardization tests have been satisfactorily completed, the serum is dispensed in 5000-unit doses with an excess of 20 per cent. The volume varies from 6 to 10 ml. If kept under proper conditions, the serum is considered satisfactory for use for eighteen months from the date of the last satisfactory test on persons.

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CHAPTER 53

PRODUCTION AND STANDARDIZATION OF THERAPEUTIC ANTIPNEUMOCOCCUS SERA

I. HORSE SERUM

Antipneumococcus sera for the treatment of pneumonia are produced by the immunization of horses against representative strains of various types of pneumococci. The sera are concentrated and purified. Their potency is determined by protection tests in mice. The amount of type-specific precipitable antibody nitrogen is also used as a measure of potency. At present, antipneumococcus horse sera of types 1, 4, and 5 only are distributed.

Antipneumococcus serum, type 1, has been distributed since 1915; later the production of sera of a number of other types was undertaken. Methods of production and standardization are based fundamentally on early experimental studies in animals of the action of immune serum in pneumococcus infection (1–5).

Strains

The strain used for the production and standardization of antipneumococcus serum, type 1, is:

Pneumococcus, type 1: "N" strain (Neufeld). Collection No. 1 (original No. 5). Received in June, 1914, from the Rockefeller Institute, which had secured a culture from Professor Neufeld of Berlin about 1910–11.

Standard strains of pneumococci of the other types have not been designated.

Maintenance.—Strains in active use are maintained in serum semisolid beef-infusion agar with frequent passage through mice to preserve their virulence.

Each new lot of serum semisolid agar (*F36C*) and of broth (*F4*) is tested before use for its ability to support satisfactory pneumococcus growth.

Pneumococcus strains that have a marked virulence for mice, such as type 1, if in active use, are passed through mice once a month; those of apparently lower virulence, once a week. Strains of type 7 may be kept at high virulence by more frequent passage (daily, Tuesday through Friday).

For animal passage use a broth culture of heart's blood or the first broth transfer of a serum semisolid agar culture from a recent mouse passage. Make a transfer of the agar culture by inoculating a tube of broth with

0.1 ml., streak a blood-agar plate (*F43A*), and incubate the cultures at approximately 35°C. for from twelve to sixteen hours. Examine the plate culture and a slide preparation of the broth culture stained by Gram's method. If no contamination is indicated, dilute the culture (see p. 684) and inject a mouse intraperitoneally with a lethal dose. At autopsy, inoculate a tube of broth (*F4*), two or more tubes of horse serum semisolid agar (*F36C*) for stock, and streak a blood-agar plate with a loopful of heart's blood. After incubation, if no evidence of contamination is indicated in the plate culture and slide preparation of the broth culture, streak a blood-agar plate with each agar culture and store the latter in the cold room. Retain for six months if no contamination is indicated in the plate cultures. Cultures from mice dying after forty-eight hours are not used.

Identification and Purity Tests.—Test the broth culture made at autopsy after every second or fourth passage, after every passage, if indicated, for solubility in sodium lauryl sulfate solution (*F73.5*). If the bacterial cells do not dissolve, repeat the test using bile. Test also for agglutination or capsular swelling reactions with antisera of the homologous and heterologous types. Occasionally examine the cultures for capsules and test for fermentation of inulin. Always make complete tests of strains not in active use before work with them is resumed.

PRODUCTION OF SERA

IMMUNIZATION OF HORSES

Horses are immunized by the intravenous injection of killed pneumococci suspended in salt solution. Usually from five to ten months, occasionally longer, are required before the serum is of sufficient potency for bleedings. A rest period of from one to three months each year, or more frequently if indicated by the condition of the horse, is advisable. The production period may extend for five years or longer.

Preparation of Cultures.—Use the first or second broth transfer from a 12- to 16-hour broth culture inoculated directly from a mouse, or from a serum semisolid agar culture of a recent mouse passage, which has been tested for purity. Prepare a subculture by pipetting 0.1 ml. of the broth or agar culture into a tube of broth, streak a blood-agar plate, and incubate both at approximately 35°C. for from twelve to sixteen hours. Examine the plate culture and a slide preparation of the broth culture stained by Gram's method. If no contamination is indicated, pipette from 1 to 3 ml., depending upon the volume, into the required number of flasks or bottles of broth, usually one or more 4-liter bottles containing 3000 ml. each. Inoculate at one time sufficient broth for a series of doses. Streak a blood-agar plate with the seed culture. Incubate the cultures at approximately 35°C. for twelve hours. Examine as previously described.

In order to obtain suitable growth with some strains of pneumococci, it is necessary to enrich the broth before inoculation by adding normal horse serum to give a concentration of from 1 to 2 per cent.

Preparation of Vaccines.—If the broth cultures were found satisfactory, remove a 1-ml. sample from each and add sufficient formalin to each bottle to give a final concentration of 1 per cent (1 ml. per 100 ml. of culture). Allow the bottles to remain at room temperature overnight. Pool the 1-ml. samples from each and, using suitable dilutions, make blood-agar poured-plate cultures. The next day make colony counts and estimate the number of pneumococci per milliliter in the pooled sample. Calculate the volume (V) in milliliters that will contain 6.25 billion pneumococci per milliliter as follows:

$$V = \frac{\text{Total number of pneumococci}}{6{,}250{,}000{,}000}$$

One milliliter of this vaccine is equal to 25 ml. of a broth culture that contains 250,000,000 pneumococci per milliliter. Siphon or pipette the culture into graduated, sterile centrifuge bottles and centrifuge until the supernatant is clear. Draw off the supernatant and discard. Suspend the cells in a small amount of salt solution. Pass the suspension through a sterile cotton filter (see p. 622) previously moistened with 1 or 2 ml. of the solution. Rinse the centrifuge bottles and filter the washings. Wash the filter with an additional 2 or 3 ml. of the solution and bring the suspension to the calculated volume. Streak a blood-agar plate with a small loopful of the suspension. Close the bottle with a sterile rubber stopper, cap with fishskin held in place by elastic bands, and immerse in a water bath. Bring the temperature to 75°C. and heat for twenty minutes. Take the temperature from a thermometer placed in a similar bottle containing salt solution. Streak a blood-agar plate and inoculate one tube of aerobic "sterility-test" broth with about 0.1 ml. of the heated suspension. No contaminating microorganisms should appear in the plate culture of the live suspension, and the plate and broth inoculated with heated suspension should remain sterile. The morning the horses are to be injected, dispense the amount required in individual doses and dilute with salt solution to 20 ml. Stock suspensions prepared in this way remain Gram-positive for two months or more. They are not used when approximately 10 per cent of the microorganisms no longer retain the stain.

The antigenic activity in horses of pneumococci killed by the action of formalin in a 1-per-cent concentration overnight at room temperature, and suspended in 1-per-cent dipotassium phosphate (K_2HPO_4) solution, pH 8.8, without heating, and of pneumococci suspended in the phosphate solution and heated at 52°–54°C. for thirty minutes (6, 7) proved satisfactory, but had no particular advantage over the method adopted.

Live Culture: Usually live culture is used only for the immunization of those horses that have failed to respond to the killed vaccine.

Vaccine Method: The first dose of live pneumococci is a suspension from 25 ml. of broth culture (amount of growth undetermined). The doses may be increased by 15 or 25 ml., depending upon the reactions induced. Prepare the broth culture as previously described. Use serum semisolid agar cultures made from six- to nine-month-old stock cultures to prepare the seed cultures. On the morning the culture is to be injected, centrifuge and suspend the cells in a sufficient volume of salt solution to give individual doses of 20 ml.

Whole Culture and Blood Method: In the whole culture and blood method, defibrinated blood from a normal horse, or preferably from one of the horses undergoing immunization against the same strain of pneumococcus, is added to the actively growing culture before the period of incubation is completed. This method is used in the immunization of some horses in which a satisfactory response to live culture prepared by the vaccine method is not obtained. Since more severe reactions are likely to occur, smaller and carefully gauged doses are required. Definite protective activity should be present in the serum of horses before they are transferred to this method.

Prepare the broth culture as for the vaccine method. After it has been incubated for eight hours, add defibrinated blood not more than four days old to give a 2-percent concentration and incubate for an additional four hours. Examine the plate culture made when the broth was inoculated and a slide preparation of the blood-broth culture. If no contamination is indicated, draw the culture through a thick cotton filter by negative pressure to remove agglutinated pneumococci and gross particles, and dispense the required amount. Inject the culture immediately after filtration.

Injection and Dosage.—Injections are made intravenously on 2 or 3 successive days with a 7-day interval between each series. The amount injected depends primarily upon the temperature and physical reactions after the previous injection and upon the general condition of the horse. The initial dose is a suspension of killed pneumococci from 2.0 ml. of the prepared vaccine. For subsequent injections of the series the dose may be increased by 2.0 ml., repeated or even reduced, depending upon the reaction of the horse. At present two schedules of injection are used. In schedule A, the dose is much diluted and given in two parts with a half-hour interval between, the first about a quarter of the total dose. The three injections of the initial series are usually reduced to two when 8.0 ml. or more of vaccine are given. In schedule B, however, the three doses are continued, the first being usually from one-third to one-fifth the last dose of the preceding series, the second the same as the last dose, and the third increased when possible.

A maximum dose of 20 ml. of vaccine may be given but in general not more than 12 or 16 ml. are injected.

Reactions.—Temperatures are taken each morning. They are also taken in case of reactions following the injection. Injections are omitted if the morning temperature is 101°F. or above, if there is other indication of infection, or if advised by the veterinarian.

Temperatures are always taken approximately four hours after the injection of live culture. A rise to 106°F. and over may occur. A temperature over 105°F. is undesirable, however, and the next dose of culture should be decreased; from 103° to 104°F. is preferred. Should the temperature continue above normal, blood is taken for culture and if pneumococci are found, immunization is discontinued.

Lesions of vegetative endocarditis, also acute diffuse and focal or embolic glomerulonephritis have been found in a number of horses undergoing immunization with live and highly virulent strains of pneumococci (8, 9). From a number of those receiving type-1 pneumococci, which developed cardiac lesions, atypical pneumococci have been isolated from the blood stream, in some instances from the synovial fluid from swollen hock joints, and from the heart lesions at autopsy (10).

Reactions manifested by trembling, dyspnea, increased pulse rate and respiration, and circulatory collapse may occur in some horses following injection. In such instances the next dose is decreased. If a reaction occurs following the small, diluted initial dose, the larger dose is omitted (see p. 65). Reactions of this type follow more frequently the injection of killed than of live pneumococci.

Bleedings.—Trial bleedings are taken before injections are commenced and at suitable intervals during early immunization. When tests show that the serum is of sufficient potency, bleedings of from 7 to 9 liters are taken seven days after the last injection. If the potency of the serum is high a bleeding is taken after only one series of injections; otherwise, after two or even three. Injections are resumed on the fifth day after the bleeding, if one series of injections is given; on the second day, if more than one series.

The period of immunization required before the potency of the serum is sufficiently high for bleedings to be taken varies widely with different horses and with the different types of pneumococci used for immunization. Horses receiving pneumococcus, type 1, are usually bled when the titer of the serum reaches 400 units—generally after from five to six months' immunization. In the case of type 2, bleedings were taken when the potency had reached 150 units or more—after from nine to ten months, occasionally a year. The highest estimated titer thus far obtained has been 1500 units for type 1; 1000 units for type 2. Horses immunized against types 4, 5, and 7 were generally bled after from five to seven months; type 8 after from eight to ten months, in two instances after five months. Experience with other types is limited but it would appear that from five to eight months' immunization is usually necessary.

TREATMENT OF SERUM

After the serum from the bleeding has been drawn off from the clot, sterility tests are made and preservative is added. The serum is concentrated and purified.

Preliminary Sterility Tests. Addition of Preservative. Filtration.—Test the serum for sterility and add preservative, at present sufficient 10-per-cent 'Merthiolate' to give a final concentration of 0.01 per cent. Remove a sample for potency tests. Should the serum be contaminated, a second test is made four days after the addition of preservative. If the retest indicates contamination, the serum is filtered at once. After filtration, samples for sterility and potency tests are taken.

Concentration and Purification. Filtration.—The serum is concentrated and purified as described in "Concentration of the Plasmas and Sera of Immunized Horses," p. 725. A 20-ml. sample is taken for potency tests from the pooled serum before and after treatment with heat and kaolin and after concentration. Due to the viscosity of the water-insoluble globulin, all concentrated preparations are diluted primarily to a total solids content of from 10 to 11 per cent. After potency tests are completed, if necessary to give the desired potency, the material may be diluted further with 0.8-per-cent salt solution containing 0.3 per cent of cresol.

Since precipitation may continue even after several months, the material is not filtered until shortly before it is dispensed. Tests for sterility and potency are made of the filtered product. If contaminated, it is refiltered as soon as possible.

STANDARDIZATION OF SERA

Antipneumococcus serum is standardized (11) by determining its protective value for white mice against the standard homologous strain—or a provisional standard—in comparison with the protective activity of a control serum. The potency of sera for which official standards have been established is expressed in units per milliliter.

The amount of type-specific precipitable antibody nitrogen is also used as a measure of potency. The methods are those described by Heidelberger (12–14).

To estimate the potency of sera subjected to heat during the process of concentration and purification, the nitrogen value should be compared with that of a sample of the standard serum heated similarly, since the amount of precipitable nitrogen is increased following such treatment (15).

For preliminary and supplementary tests of sera of all types agglutination tests with pneumococcus suspensions and precipitation tests with specific carbohydrates are employed.

Standard Control Sera

For standard control sera, unconcentrated material of high titer not less than four months old should be selected.

The unit value of the present standard control serum, type 1, was based on the potency of the Federal standard serum P11 (a bivalent serum of types 1 and 2) and on the international unit value for type-1 serum, namely 0.0886 milligrams of the dried serum prepared by Hartley as a standard and adopted by the Permanent Commission on Biological Standardization of the League of Nations (16). No unit values have been adopted for sera of types 4 and 5.

PROTECTION TESTS

Different amounts of each serum are mixed with the test dose of culture and injected intraperitoneally into mice. In each test, as a basis of comparison in estimating the potency of the test sera, mice are injected with mixtures of different amounts of the control serum and the same test dose of culture. As a culture control the number of microorganisms per milliliter are determined from blood-agar poured-plate cultures made at the time of the test, and the virulence is tested by injecting mice with different doses of the culture, one of which is the minimum lethal dose.

The smallest number of microorganisms of maximum virulence that will kill in forty-eight hours a mouse weighing between 18 and 22 grams is considered the minimum lethal dose of a pneumococcus culture.

Culture.—The first broth transfer from a heart-blood broth culture, occasionally the direct culture, from a mouse dying within forty-eight hours after receiving approximately 100 M.L.D. of pneumococci is used for the test. The culture is incubated in a special broth (*F4*) at 35°C. for sixteen hours.

The broth selected for use in protection tests is one found by preliminary tests to be especially favorable for growth. Different lots of broth vary in this quality. Chemical analyses of infusions of beef skeletal muscle and of different lots of broth made with them have confirmed previous observations that a relatively high content of fermentable and other reducing substances is essential for the growth activity of the pneumococcus (17). The wide variations with different lots of meat in the amount of these substances, probably for the most part muscle sugar, are dependent apparently upon the animal's condition when slaughtered and the length of time and manner of storing the meat.

In order to obtain satisfactory growth of certain strains for protection tests (from 200 to 300 million or more pneumococci per milliliter), it has been necessary to enrich the broth before inoculation by adding normal rabbit serum to give a 0.5-per-cent concentration.

Test Dose of Culture.—The test dose of culture is determined for each strain of pneumococcus and is expressed as the number of pneumococci. It is selected on the basis of protection tests in which different amounts of culture are used as a test dose and should be whenever possible in the range in which the ratio of the dose of culture to the protecting dose of serum is constant. Standardization tests with the optimum test dose should give uniform results in repeated tests.

The optimum test dose of culture varies markedly with strains of different types (18). For example, a test dose of approximately 25,000,000 pneumococci, or 0.075 ml. of the broth culture of the type-1 strain, No. 1, contained in 0.5 ml., is used in standardization tests; approximately 40,000 pneumococci or 0.0001 ml. of the type-4 strain, No. 36364.

The Test

Healthy white mice weighing between 18 and 22 grams are used.

Preparation of Dilutions.—For general procedure, see "General Bacteriologic Technic," p. 24.

Serum: Three doses or more of each serum are tested. The ratio of successive doses should not be more than 2. Dilute the test and standard control sera in salt solution so that the desired amounts of serum are contained in 0.5 ml. Thus, if the dose of serum to be injected is 0.005 ml., prepare a hundred-fold dilution. After all the containers have been filled from a burette (factory certified) with the desired volumes of salt solution, add the required amount of serum to the first container with a 0.5 or 1-ml. volumetric pipette. Because of the high viscosity of the concentrated preparations, rinse the pipette twice with the dilution. With a graduated pipette mix thoroughly by drawing up the solution and discharging it several times; transfer the required amount to the next container. With a different pipette mix thoroughly the contents of any container that holds material to be used for a further dilution and with the same pipette transfer the required amount. Whenever possible, plan all dilutions so that not less than 0.5 ml. or more than 1 ml. will be pipetted.

Culture: Dilute the culture in broth so that the desired amounts are contained in 0.5 ml. After the dilution for the test dose has been prepared similarly to those of serum, mix thoroughly and dispense in stoppered containers, one for every ten mice to be injected. Dilute the culture for the control tests. Pipette into the necessary number of tubes the required volume of broth, 2 ml. into the first and 4.5 or 9.5 ml. into the succeeding tubes, depending upon the dilutions of culture to be prepared. Add 0.5 ml. of culture to the first tube. With a fresh pipette mix thoroughly by drawing up the suspension and discharging it several times; transfer 0.5 ml. of this dilution to the next tube. Use a fresh pipette for each dilution.

Culture Control.—To determine the number of pneumococci, three dilutions of culture are selected, on the basis of previous tests, which contain approximately 3, 30, and 300 microorganisms in 0.5 ml., numbers which are readily counted. Pipette into Petri plates 0.5-ml. amounts of the dilutions in duplicate, then pour into each melted beef-infusion agar (*F35A*) to which defibrinated normal horse blood not more than one week old has been added immediately before to give approximately a 10-per-cent concentration. Mix well by a gentle rotary motion. Incubate at approximately 35°C. for forty-eight hours and count the colonies. Each pneumococcus colony is assumed to represent the growth of one microorganism.

For the virulence control, inject three mice with approximately 1, 3, and 30 M.L.D. respectively of the culture. With cultures of high virulence these doses are generally 0.000,000,005, 0.000,000,01, and 0.000,000,1 ml. contained in 0.5 ml.; with those of lower virulence, larger amounts.

Injections. Observation of Animals. Necropsies.—All injections are made intraperitoneally and are completed within one hour from the time the culture is diluted. See "The Use of Experimental and Test Animals," p. 77. Use tested 2-ml. syringes and 24-gauge needles, one-half of an inch long. Begin with the smallest dose. Immediately after injection mark each mouse for identification. Inject first the mice for the virulence control. Record the time of injection. Inject next the mice which receive mixtures of the standard control serum and culture and then the test-sera mixtures. To inject the mixtures draw into the syringe 0.5 ml. of the culture dilution, then 0.5 ml. of serum dilution, and inject immediately. If ten or more mice are used for one dilution of serum, take a separate syringe for each dilution.

Inspect mice three times daily, removing those that have died, at 9:00 a.m., 5:00 p.m. and 12:00 midnight for seven days; when indicated, at 1:00 p.m. also. In warm weather the removal of dead animals at 5:00 a.m. in addition may be desirable. Transfer dead mice to paper bags, recording on the latter the necessary data, and place in a low-temperature chamber. Unless contraindicated, discharge on the eighth day all mice that survive.

When autopsies are to be made, perform them as soon as practicable (p. 84), and make cultures and slide preparations from the heart's blood. Autopsy all mice used in virulence control tests. In final tests of sera in which groups of ten mice are used, autopsy all that die unless the number in a group exceeds six, then only six. In all other tests, autopsies are omitted unless additional information is desired, such as in experimental tests or when animals die out of order or infection is suspected.

Interpretation of Test and Estimation of Potency.—Mice inoculated with serum-culture mixtures and surviving for ninety-six hours are considered protected by the amount of serum with which they were injected. Those inoculated with culture for the virulence control tests should die within forty-eight hours. If contaminating microorganisms other than *Bacterium coli* are isolated at autopsy or if no pneumococci are present, the mouse is ignored in the interpretation of the test.

With serum-culture mixtures of some types, many of the mice die on the fifth and sixth days after injection. Mice inoculated with such mixtures are therefore not considered to have survived unless they live for seven days. In the virulence control tests mice that receive cultures of certain types frequently survive for forty-eight hours, but die within four days.

No test is considered satisfactory unless at least one dose of the test serum and one dose of the standard serum protect 50 per cent of the mice for four days or longer and unless the next smaller dose fails to protect 50 per cent. The ratio of successive doses should not exceed 2.

The potency of antipneumococcus serum is estimated by determining the relation of the protecting dose of the test serum to that of the standard control serum. The estimated amount of serum that protects 50 per cent of the mice for four days against the test dose of culture, under the conditions of the test, is considered the protecting dose of the serum. Thus, if the protecting dose of the test serum is 0.0025 ml. and that of the standard control serum 0.01 ml., the estimated potency of the test serum is four times that of the control serum, or 4000 units per milliliter, based on a value of 1000 units per milliliter for the standard control serum.

Since the 50-per-cent end point is estimated from a limited number of doses, it is more economical to employ some form of statistical graduation of the data, including neighboring doses on either side of the protecting dose. The doses of serum should be spaced equally far apart on a logarithmic scale, that is, in a geometric progression, and equal numbers of mice should be used for each dose. The best of the generally applicable statistical methods appears to be the conventional use of *moving averages* and an interpolation between values nearest the 50-per-cent end point. The actual calculation may be simplified by use of relation (6) on p. 152 by substitution of the actual values for n, k, and d. In the present system $n = 10$, $k = 2$, and $d = \log 2 \cong 0.301$. The relation reduces to

$$\begin{aligned} \log m &= L_a + d\tfrac{1}{2} + d\left(\frac{10 - r_a - r_{a+1}}{r_{a+2} - r_a}\right) \\ &\cong L_a + 0.301\left(\tfrac{1}{2} + \frac{10 - r_a - r_{a+1}}{r_{a+2} - r_a}\right) \end{aligned} \tag{6.3}$$

The *trial function* $= r_a + r_{a+1} + r_{a+2} - 10$ which should lie in the interval (r_a, r_{a+2}) for interpolation to result.

The potency of the finished preparations and of concentrated preparations before dilution is based on similar results obtained in two satisfactory tests in each of which ten mice are used for each dose of serum. In preliminary tests of bleedings frequently one test in which five mice are used for each dose is adequate for an approximation of the potency.

PRECIPITATION TESTS

Varying dilutions of the sera to be tested and of the standard control serum are mixed with equal volumes of an optimum dilution of specific carbohydrate (19, 20). The tubes containing the mixtures are placed in the cold room overnight and the readings made the following morning in comparison with the control-serum mixtures.

Carbohydrate Solution.—A 0.1-per-cent solution of the specific carbohydrate is prepared in salt solution and 10-per-cent 'Merthiolate' is added to give a 0.01-per-cent concentration. The hydrogen-ion concentration of the solution should be approximately pH 7. The dissolved preparation is filtered through a Mandler filter candle and stored in the cold room.

The Test

Dilutions.—The dilutions are made in salt solution.

Serum: Usually from 6 to 8 dilutions in geometric progression with increments of 100 per cent are tested, the range depending upon the potency of the material. If a closer approximation of the titer is desired, the increments may be 50 per cent or less in the range where an end-point is expected. If a precipitate is in suspension in the serum, centrifuge the serum before making dilutions.

Carbohydrate: From the 0.1-per-cent stock solution of carbohydrate, prepare the required dilution for each test.

In determining the optimum dilutions of carbohydrates of the different types of pneumococci, a test is set up with several identical series of from 6 to 8 dilutions of a serum in a range selected on the basis of the estimated potency of the serum. Different dilutions of carbohydrate are added to each series and the reactions noted after standing in the cold room overnight. The dilution of carbohydrate that gives definite and evenly suspended particles and well-graded reactions with the different serum dilutions is selected as the optimum. This dilution varies with carbohydrates of the different types. For example, at present a 40,000-fold dilution of the type 1 and a 160,000-fold dilution of the type 7 are used.

Mixtures.—Use unplugged, clear tubes (75 by 11 mm.). Beginning with the greatest dilution of serum, mix each in turn and pipette 0.3 ml. into a tube. Add 0.3 ml. of the carbohydrate dilution, then 0.4 ml. of salt solution. When a large number of sera are to be tested, set up a separate series of control serum-carbohydrate mixtures for every six. Shake the racks well and place them overnight in the cold room before making readings.

Readings and Estimation of Titer.—Record the degree of the reactions as described in "General Bacteriologic Technic," p. 22. Read the reactions of the standard control serum first, then compare the reactions of the test sera with those of the control and record like reactions similarly. Since excessive shaking affects the suspensions, not more than six test sera should be compared with one set of control-serum mixtures.

In estimating the titer of a serum all the reactions of both the test and control sera are considered. The titer of a test serum is recorded as equal to or a fraction or multiple of that of the standard control serum. If no end-point of precipitation is obtained, the test should be repeated with greater or smaller dilutions of serum as indicated.

The simple procedure described has proved very useful as a preliminary test for the titration of serum and in general the results of these tests and protection tests closely parallel each other. With the concentrated preparations, however, the reactions are more difficult to read and the results therefore less definite.

AGGLUTINATION TESTS

Varying dilutions of serum are mixed with equal volumes of uniform bacterial suspensions of pneumococci of the homologous type. In certain tests, suspensions of heterologous types are also included. The tubes that contain the mixtures are incubated in a water bath or incubator at from 35° to 37°C. for two hours.

The Test

Serum Dilutions.—The general procedure and scheme of dilutions is that given under "Precipitation Tests."

Bacterial Suspensions.—Prepare the suspensions immediately before they are to be added to the serum dilutions. Use broth cultures prepared as described on p. 678. Centrifuge the culture until the broth is clear and pour off the supernatant carefully if it is to be used for precipitation tests. Add salt solution slowly to the cells and mix until a uniform suspension is obtained. Twist a thin layer of nonabsorbent cotton around the tip of a pipette to act as a filter, and with the pipette remove the suspension from the centrifuge cup, discard the cotton with forceps, and transfer the suspension to a tube. Dilute the suspension further with salt solution until it is similar in turbidity to glass standard No. 4. Test each suspension for solubility in sodium lauryl sulfate solution. If the bacterial cells do not dissolve, repeat the test using bile.

Whole broth cultures have also proved satisfactory in tests of agglutinative activity. The supernatant broth from the centrifuged cells may be used to determine precipitative activity.

Mixtures.—The general procedure for preparing the mixtures is that given in "General Bacteriologic Technic," p. 20. Shake the mixtures well and incubate at 35°–37°C. for two hours.

Readings and Estimation of Titer.—Read and record the degree of reaction as described on p. 21. Should the cells fail to dissolve, the entire test is considered indeterminate.

In estimating the agglutinative titer of a serum all the reactions of both the test and control sera are considered. The titer of a test serum is recorded as equal to or a fraction or multiple of that of the standard control serum.

PREPARATION FOR DISTRIBUTION

After the sterility and potency tests have been satisfactorily completed, the serum may be prepared for distribution. Antipneumococcus serum, type 1, is dispensed in 25,000-unit doses with an excess of 20 per cent, contained in a volume of from 7.5 to 11 ml. Antipneumococcus sera of types 4 and 5 are dispensed in an arbitrarily selected dose of 20 ml. If kept under proper conditions, the sera are considered satisfactory for use for eighteen months from the date of the last satisfactory test.

In each package of serum is included a vial containing 1 ml. of a 1:10 dilution of normal horse serum for use in sensitivity tests. Sterile physiologic salt solution is also distributed in 10-ml. amounts for use in preparing the dilution for the intracutaneous test of sensitivity, for rinsing water from syringes and needles that have been boiled, and for diluting the serum for the preliminary injection.

PREPARATION OF DILUTED HORSE SERUM

Serum from a normal horse in good condition is used. The diluent is salt solution containing cresol in a 0.3-per-cent concentration.

To the exact amount of salt solution, sterilized in bottles graduated at the proper volume, add with aseptic precautions sufficient cresol to give a 0.3-per-cent concentration. Mix thoroughly by rotating the bottle. Replace with water the loss by evaporation during sterilization. Add the required volume of serum with a pipette or a siphon, depending upon the volume, and mix thoroughly. Make the usual tests for sterility. Filter shortly before required and when the sterility tests are satisfactorily completed dispense in 1-ml. volumes. If kept under proper conditions, the diluted normal serum is considered satisfactory for use for two years after the date of preparation.

PREPARATION OF PHYSIOLOGIC SALT SOLUTION

The salt solution is prepared immediately before it is dispensed according o the standard formula (*F64*) except that it is passed through a Mandler ilter candle and then sterilized by autoclaving at 121°C. for thirty minutes.

It is dispensed in 10-ml. volumes and if kept under proper conditions is considered satisfactory for use for eighteen months from the date of preparation.

II. RABBIT SERUM

Antipneumococcus sera for the treatment of pneumococcus infections are produced in rabbits against a number of types of pneumococci. The serum, concentrated and unconcentrated, is processed by the method of Goodner, Horsfall, and Dubos (21). Standardization is, when possible, by the protection test in mice, supplemented by determinations of antibody nitrogen according to the methods of Heidelberger (12–14).

Strains

Maintenance.—The representative strains are maintained in rabbit-serum semisolid agar (*F36C*). Each culture is passed through a mouse at least once a month. Certain types are maintained by serial passage in mice. For procedure, see p. 677. The type-3 strain used for live culture vaccine is kept in the dry state.

Identification and Purity Tests.—Examine daily the blood-agar plate inoculated at autopsy for seventy-two hours for the type of growth and evidence of contamination. Test the broth culture for solubility (p. 18) and for specific agglutination reactions. If contaminants are present or if unsatisfactory results are obtained in the serologic test, discard the serum semisolid agar cultures. If more than one kind of pneumococcus-like colonies are noted on the blood-agar plate, make representative fishings to broth and test each culture for solubility and specific agglutination.

Agglutination test: Test the sera to be used for typing for specific activity and for cross reactions with all other types. Determine a suitable dilution for each. Prepare pools containing sera of several types in the dilution required. Make a macroscopic agglutination test (p. 19) with the mouse-heart-blood broth culture. Test also in the univalent sera of the types that make up the pool in which a reaction is obtained.

PRODUCTION OF SERA

Preparation of Cultures.—Formalinized, heated vaccine is used for the immunization of rabbits with all types of pneumococci in use except types 3 and 19. For the latter, unheated formalinized vaccine is used. For preliminary immunization with type 3, a live-culture vaccine may be given instead of the formalinized suspension.

Formalinized, Heated Vaccine: Inoculate a tube of infusion broth (*F5.5*) from the rabbit-serum semisolid agar culture and incubate for from eight to ten hours at 36 C. Streak a blood-agar plate and inoculate a flask of the same infusion broth taking care not to transfer any of the agar from the semisolid medium. Incubate for from eight to ten hours at 36°C. Streak a blood-agar plate. Examine a slide preparation stained by Gram's method. If no contamination is indicated, inoculate flasks of infusion broth, previously warmed to incubator temperature, using 3 ml. of culture for every 100 ml. of medium.

Incubate the cultures for five hours at 36°C. Streak a blood-agar plate and add formalin to a concentration of approximately one per cent. Transfer to 500-ml. centrifuge bottles and let stand at room temperature overnight. Centrifuge and draw off the supernatant. Make a concentrated suspension of the microorganisms in salt solution and filter through a sterile cotton filter. Place a sterile rubber stopper in each bottle and cap with fishskin. Submerge the bottles in a water bath and heat at 75°C. for fifteen minutes. As a temperature control insert a thermometer into a rubber stopper in a bottle of water equal in volume to the largest amount of vaccine. Cool quickly with running water. Add salt solution until the turbidity equals glass standard No. 6. Redispense in 50-ml. bottles. From one bottle of the redispensed antigen make a macroscopic agglutination test. Examine a slide preparation stained by Gram's method. If the results of serologic tests and tests of purity are satisfactory and the majority of the cells are Gram-positive, the vaccine is suitable for use.

Formalinized Vaccine: Prepare by the same method as for the formalinized, heated antigen. After the formalin is added incubate the flasks for one hour at 36°C. Transfer to 500-ml. centrifuge bottles and let stand at room temperature overnight. Centrifuge and draw off the supernatant. Resuspend the cells in salt solution to which 0.5-per-cent formalin has been added, and store in the cold. Dilute a sample of the vaccine with salt solution until the turbidity equals that of glass standard No. 6. Record the dilution factor. Immediately before use, centrifuge a measured amount of the vaccine, decant the supernatant, and resuspend the cells in salt solution to equal glass standard of turbidity No. 6, using the dilution factor previously determined. Examine a slide preparation stained by Gram's method. Make a macroscopic agglutination test. If the agglutination reaction is specific and the majority of the cells are Gram-positive, the vaccine is satisfactory for use.

Live Culture, Type 3: Inoculate a tube of rabbit-blood broth from the dried culture. Maintain the blood-broth transplant as the stock culture for not more than one month. Inoculate tubes of infusion broth (*F5A*) from the blood-broth culture and incubate overnight at 36°C. Streak a blood-agar plate. Examine a slide preparation stained by Gram's method. If no contaminants are seen inoculate flasks containing the required amount of infusion broth and incubate for five hours at 36°C. Transfer the culture to centrifuge bottles and test for agglutinative activity the small amount of culture remaining in the original flask. Centrifuge the culture and draw off the supernatant. Resuspend the cells in an amount of salt solution equal to approximately five per cent of the original culture. Filter the suspension through small cotton-plugged filters. Dilute until the turbidity equals that of glass standard No. 6. Streak a blood-agar plate and make a solubility test. Examine a slide preparation stained by Gram's method. If the majority of the cells are Gram-positive and the results of the tests satisfactory, the vaccine is used immediately.

IMMUNIZATION OF RABBITS

Selection of Rabbits.—When the animal is received from the stock animal quarters, it is weighed and examined for any evidence of nasal discharge, ear mange, and diarrhea. Only rabbits in good condition and weighing preferably not less than 2500 grams are used.

Injection and Dosage.—Injections are given intravenously. For the preliminary immunization, the rabbits receive from six to eight series. A series consists of a daily dose on three consecutive days with a five-day interval between series. The dose of vaccine is gradually increased from the first dose of 0.2 ml. to a maximum dose of 3.0 ml. When the titer of the serum is satisfactory and bleedings are begun, only one dose a week is given. One dose is given the day following bleeding and another one week later. Other schedules of immunization have also been found to be satisfactory.

Bleedings.—At the end of from six to eight series and at intervals of two weeks thereafter, 40 ml. of blood are taken from the heart. The rabbits are bled on the sixth or seventh day after the preceding dose. When five successive bleedings have been taken, one is omitted. Bleedings are also omitted at any time if the rabbit loses weight between bleedings or develops symptoms of infection, such as difficulty in breathing or diarrhea. Should the loss of weight continue, immunization is stopped, the animal is rested or bled out, and the serum used for diagnostic tests.

Technic of Bleeding: The bleedings are taken by a team of workers, one of whom does the bleeding while the other prepares the next rabbit.

Remove the crocks of feed from the cages the afternoon of the previous day. Weigh the rabbit and record the weight on the immunization and bleeding record cards. Tie the animal securely on the rabbit tray and clip the hair from the chest with an electric clipper equipped with close-fitting surgical blades. Carry the tray to the bleeding room where the bleeding is taken from the heart with aseptic precautions into a 200-ml. bottle with the aid of a suction pump (200–300 mm. Hg.). Immediately slant the bottles containing the blood on a tray. Allow them to remain undisturbed until the clot is firm and the serum begins to separate. Remove to the serum-processing room.

TREATMENT OF SERUM

Preliminary Treatment.—With aseptic precautions replace the bleeding connections in the bottles with sterile gauze-covered cotton plugs. If the titer of the bleeding is to be determined, withdraw a 2- or 3-ml. sample. Rim with a sterile 1-ml. pipette any clots that do not separate from the wall of the bottle. Store the bleedings in the refrigerator overnight. The following day remove the serum from the clot with a 50-ml. pipette and transfer to a 50-ml. centrifuge tube. Centrifuge to free from blood cells. If any sera are unusual in appearance, for example, are bile-stained or contain precipitates, place them in separate containers. Pool the remaining sera according to the approximate titer as indicated by the precipitation test with the specific carbohydrate.

As a test of sterility, add 0.5 ml. of each pool of 250 ml. or less to 20 ml. of horse-infusion agar semisolid medium (*F32B*). Cap the bottles of serum with fishskin. Place in a metal tray and totally submerge in a water bath. As a temperature control, a thermometer graduated in 0.1°C. is inserted into a rubber stopper in a bottle of water equal in volume to the largest amount of serum. Heat to 56°C. and hold at that temperature for thirty-five minutes. Store the bottles in the cold room overnight. As a preservative add phenol in ether solution to give a final concentration of 0.2 per cent, and 'Merthiolate' to give a final concentration of 0.005 per cent (22). Attach a label that suitably identifies the serum and store the serum in the cold room for an indefinite period to allow precipitates to settle. If growth is obtained in the sterility-test medium, make a retest (1 ml. in 80 ml. of horse-infusion agar semisolid medium (*F32B*)) and, should contamination be indicated, filter the serum immediately.

Final Processing.—Pool the heated sera and clarify by passage through infusorial earth followed by filtration through a final Mandler filter candle. The filtered serum is tested for sterility and either returned to the cold room for further precipitation or concentrated, see p. 728. Before distribution, all sera are processed according to the method of Goodner, Horsfall, and Dubos (21).

Place dry kaolin (2.5 grams to 100 ml. of serum) in a sterile 500-ml. centrifuge bottle with a cotton plug. Fill the cup with sterile salt solution and mix well. Allow to stand in the cold for about twenty-five minutes and decant the greater part of the supernatant containing the finer particles of kaolin. Spin lightly in the centrifuge and decant the remaining supernatant. Sterilize the kaolin in the cup by autoclaving.

Add the serum to the sterilized kaolin, mix thoroughly with a sterile glass rod, and allow to stand overnight in the cold. Centrifuge the mixture at 2400 r.p.m. for one hour, preferably in a refrigerated centrifuge, and filter first by vacuum through a preliminary Mandler filter candle or through infusorial earth in a Büchner funnel and finally by pressure through a final filter candle or a Seitz type filter.

STANDARDIZATION OF SERA

Agglutination and precipitation tests are used to follow the response of the individual rabbits to immunization. The activity of the serum from the first and second bleedings of each rabbit and from every third bleeding thereafter is determined. When there is an apparent drop in titer, tests are made at shorter intervals. As a preliminary indication of the potency of the pooled lots of sera the amount of antibody nitrogen is determined. Protection tests and antibody nitrogen determinations are made on the lots of serum for distribution. With some types of pneumococci, usually those of low virulence for mice, it is difficult or impossible to obtain satisfactory quantitative results in the protection test. The final protection tests are made on the filled reheated product.

Agglutination Tests.—See "Standardization of Antipneumococcus Sera (Horse)," p. 688.

Precipitation Tests.—Determine the appropriate dilution of the specific carbohydrate by preliminary titration with sera of high and low titers.

To 0.2 ml. of serum or serum dilution add 0.3 ml. of salt solution and 0.5 ml. of the previously determined dilution of type-specific carbohydrate, shake and read immediately by artificial light, and record. Incubate at 36°C. for two hours. Place in the cold room overnight. Note and record the character of the precipitate (21).

Protection Tests.—See "Standardization of Antipneumococcus Sera (Horse)," p. 683. Rabbit-serum dilutions are made with "Mohr" pipettes recalibrated by a precise method to deliver the required amount between graduations with a tolerance of a 2-per-cent error.

Antibody Nitrogen Determinations.—The methods of Heidelberger are followed (12–14).

PREPARATION FOR DISTRIBUTION

As soon as the sterility tests on the processed serum are satisfactorily completed the serum is prepared for distribution. Following the satisfactory completion of the sterility tests after filling, the vials of serum are immersed to the neck in water and heated at 56°C. for fifteen minutes. A label bearing the pneumococcus type and lot number is placed on each vial.

Test for Pyrogen.—In addition to the usual animal tests, each lot of serum for distribution is subjected to a thermal test in rabbits.

Determine and record the temperature of each of three rabbits. Inject each intravenously with 2 ml. of the lot of serum to be tested. One hour later again determine and record the temperature of each.

If the average of the temperatures of the three rabbits indicates a rise of more than 0.5°F., or if one rabbit shows a rise of 1°F., the test is repeated with three additional rabbits. The serum is not considered satisfactory for therapeutic use if on retest two or more rabbits show a rise of 1°F. or more.

In each package of serum is included a vial containing 1 ml. of a 1:10 dilution of normal rabbit serum for use in tests of sensitivity. Sterile physiologic salt solution in 10-ml. amounts is distributed as in the case of the horse serum.

PREPARATION OF DILUTED NORMAL RABBIT SERUM

Pooled sera from normal rabbits in good condition are used. The serum is heated in a water bath at 56°C. for thirty-five minutes. It is diluted according to the method for "Preparation of Diluted Horse Serum," p. 689.

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CHAPTER 54

PRODUCTION AND STANDARDIZATION OF ANTIMENINGOCOCCUS SERA

Multivalent antimeningococcus sera for the treatment of meningococcus meningitis and meningococcemia and for diagnostic use are produced by the immunization of horses against standard strains representative of the main serologic groups of the meningococcus. The sera are concentrated and purified. Their potency is determined by protection tests in mice, supplemented by agglutination and precipitation tests. As an aid in the evaluation of the serum, strains from current meningococcus infections are classified to determine the incidence of the different serologic groups, and the activity of the serum against strains of special interest is studied.

Antimeningococcus serum was first produced in this laboratory in 1916; by the present method with six standard strains, in 1918. The method with minor modifications has been continued in use on the basis of results of laboratory and clinical studies (1–12). A limited supply of univalent serum is maintained. The production of serum in rabbits has not been undertaken routinely since the horse serum has been shown to have high potency and broad valency. However, comparative tests of rabbit and horse sera are made from time to time.

Strains

Six standard strains are used in the production and standardization of serum: three group I, two group II, and one group IIa. Group I includes groups I and III of Gordon and Murray's classification (13). Group IIa designates "encapsulated group-II" strains (12, 14). See p. 707.

Group I. Collection No. 39 (original No. 79 B). Received about 1916 from the Rockefeller Institute.

Group I. Collection No. 69 (original No. W 60 B). Received in 1918 from the National Institute of Health, Washington, D. C.

Group I. Collection No. 68 (original No. W 30 B). Received in 1918 from the National Institute of Health, Washington, D. C. Originally classified as group III.

Group II. Collection No. 36 (original No. 46 B). Received in 1916 from the Rockefeller Institute.

Group II. Collection No. 38 (original No. 1 B). Received about 1916 from the Rockefeller Institute.

Group IIa. Collection No. 3855. Isolated in 1938 at the Children's Hospital, Buffalo, and received for classification and study.

In addition, group-I strain collection No. 37 (original No. 10 B) received in 1916 from the Rockefeller Institute, originally classified as group III, is used in standardization tests. Other strains of the different groups, and atypical strains from various sources are maintained for use in special tests of sera and in experimental studies. Recently isolated strains are added to this collection from time to time.

Maintenance.—Strains in active use are maintained on glucose serum agar slants (*F34C*) and in the dry state; other strains in the dry state only. Transfer the cultures on glucose serum agar every forty-eight hours and maintain at a temperature of 34°–36°C. Renew preparations of strains in the dry state every two and one-half to three years.

Three or more preparations of each strain suspended in broth with rabbit blood are frozen and dried according to the method of Swift (15) and stored at room temperature, see "Collection of Type Cultures," p. 90. Freshly isolated strains are frozen and dried as soon as practicable.

Identification and Purity Tests.—Glucose serum agar cultures: At least once each week, make slide preparations stained by Gram's method. Every six months, plate each strain on blood agar (*F43A*). Fish at least three colonies and test the agglutination and precipitation reactions of each with the homologous and heterologous univalent group sera. Use the immune-serum-agar-plate method for the precipitation tests, see p. 709. Test the cultures for fermentation of maltose, glucose, sucrose, and lactose. Inoculate tubes of beef-infusion ascitic-fluid semisolid agar containing phenol red indicator and the carbohydrate (*F39B*), and incubate for forty-eight hours. Make readings at the end of twenty-four and forty-eight hours, in case of doubtful reactions also after seven days, and record the results as 2+ (strongly acid reaction); +; ±; alk. (alkaline reaction); and − (no reaction). Meningococcus cultures ferment maltose and glucose but not sucrose and lactose. Freshly isolated cultures occasionally show no fermentative activity, or ferment maltose only; very rarely, glucose only. Typical fermentation reactions are generally obtained after continued cultivation. Plate and test similarly cultures not in active use before work with them is resumed.

PRODUCTION OF SERA

IMMUNIZATION OF HORSES

Cultures of the six standard strains, grown on glucose serum agar slants, are suspended in salt solution, pooled, and injected intravenously. Live cultures are used, and the doses are increased gradually. Bleedings are taken after six months if the serum has reached the required potency. A rest period of from two to three months each year, and more frequently if indicated by the condition of the horse, is advisable. For the technic of injections and bleedings, see "Care and Use of Large Animals," p. 61.

Preparation of Cultures.—Make transplants from 24- or 48-hour cultures of the required strains to glucose serum agar slants (*F55.2*) (150- by 19-mm. tubes) and incubate for from sixteen to eighteen hours. If the growth is abundant and there is no evidence of contamination, prepare the suspensions. Add 3 ml. of salt solution to each culture. Loosen the growth carefully with a loop and stir until a uniform suspension is obtained, taking care not to break the agar. Pass the suspensions through a sterile cotton filter (see "General Instructions," p. 622), previously moistened with a volume of salt solution equal to 1.5 ml. for each culture. Then pass through 1.5 ml. of salt solution for each culture. Pipette the dose prescribed for each horse into a separate sterile bottle and add sufficient salt solution to bring the volume to 20 ml. If severe reactions are induced, dilute subsequent doses to 50, 100, or even 200 ml.

Injections and Dosage.—Injections are made into the jugular vein. Doses are given on three successive days with a 7-day interval between series.

The suspensions are injected as soon as prepared. To avoid insofar as possible severe reactions, the required dose is divided and two injections are made an hour apart. Duplicate sets of cultures are inoculated on the preceding afternoon and the suspensions for each injection prepared separately immediately before the injection. The doses have, at times, been given in single injections.

No definite schedule of doses can be followed, since the reaction induced in different horses varies widely. Each dose is based largely on the temperature and physical reactions after the previous injection and on the general condition of the horse. Immunization is begun with very small doses of the living cultures, not more than 0.2 ml. of the pooled suspension on the first day. During the early series the doses may be increased by from 0.2 to 0.6 ml. In later series the increase is greater. In general, maximum increases have varied from 2 to 6 ml., maximum doses, from about 15 to 30 ml. Usually the first dose of a new series is the same as the last dose of the preceding series. On resuming injections after bleedings, from one-half to three-quarters of the previous dose is given. Care is taken not to increase the dose so rapidly that the general condition of the horse is impaired.

Reactions.—Temperatures are taken every morning and also four hours after the second injection of each dose. Additional readings are made if temperatures of 104°F. or over are recorded. The aim is to give a dose that will induce a reaction of from 102° to 104°F. with a return to normal by the following morning. If the temperature remains over 101°F., treatment is postponed until it returns to normal, and the same or a smaller dose is given at the next inoculation. If there is evidence of infection, the injections are suspended and a sample of blood is taken for culture. Treatment is discontinued if meningococci are recovered. Vegetative endocarditis has developed in a number of horses in the course of immunization (16, 17).

Physical reactions, such as trembling, increased pulse and respiration, dyspnea, etc., are carefully noted and recorded. As far as possible, the doses are graded to avoid such reactions.

The second injection of the divided dose is omitted if a marked reaction has occurred, and the same or a smaller dose is given at the next inoculation.

Bleedings.—Trial bleedings are taken immediately before the first series of injections, before every fourth series, and, when the results of tests indicate the serum is approaching the required potency, before every other series. Bleedings of from 7 to 9 liters are taken after six months' immunization if the titer is satisfactory. They are taken on the sixth day after the last inoculation. Inoculations are resumed on the fifth day after bleeding, and two series are usually given between bleedings.

TREATMENT OF SERUM

After the serum from a bleeding has been drawn off from the clot, sterility tests (see p. 830) are made and preservative is added. The material is concentrated and purified. Later it is filtered and dispensed for distribution.

Preliminary Sterility Tests. Addition of Preservative.—After the required media are inoculated for sterility tests, 3 ml. of cresol per liter (0.3 per cent) are added as preservative. The cresol is emulsified in a volume of distilled water equal to 2 per cent of the total quantity of serum and the emulsion is added very gradually to the serum which is stirred by an electric stirrer. About 10 ml. of the cresolized material are withdrawn for potency tests, and the serum is stored in the cold room. Should the sterility tests indicate that the serum is contaminated, a retest is made. If the second test shows contamination, the serum is filtered at once.

Concentration and Purification. Filtration.—The serum is concentrated and purified according to the procedure in "Concentration of the Plasmas and Sera of Immunized Horses," p. 725. Sera that approximate the standard control serum in precipitative titers are selected and pooled in lots of about 40 liters for concentration. A 20-ml. sample is taken from the pooled serum before and after concentration for potency tests. All concentrated preparations are diluted to a total solids content of not more than 9 per cent. Two or three days after concentration is completed, a sterility test is made of the concentrated material. Concentrated products of high titer may be further diluted with 0.8-per-cent salt solution containing 0.3 per cent of cresol.

Concentrated preparations are usually not filtered until shortly before dispensing, since precipitation may continue to occur even after several months' storage. Products found to be contaminated in the preliminary tests are filtered promptly and tests of sterility made. As in the case of the unfiltered serum, should the sterility tests indicate that the product is contaminated, a retest is made. If the second test shows contamination, it is refiltered immediately.

STANDARDIZATION OF SERA

Multivalent antimeningococcus sera are standardized by determining their protective activity for mice against standard or provisional standard strains of groups I, II, and IIa, in comparison with that of a standard control serum. For preliminary tests of sera, precipitation tests with ultrafiltered residues of broth culture filtrates are employed. The titer and valency of the sera are further controlled by agglutination tests with the standard strains and occasionally with freshly isolated cultures. Experimental studies of other methods have aided in gauging the relative potencies of different sera (10, 11, 18).

Standard Control Serum

For the standard control, an unconcentrated serum not less than four months old and obtained from a horse under immunization for at least six months is selected on the basis of its protective, precipitative, and agglutinative activity. It is used as a standard of comparison in all three methods of evaluation and represents the minimum standard of potency.

The control serum may be preserved by freezing and drying (19). It is dispensed for drying in 10- and 25-ml. amounts. The resuspended serum may deteriorate in titer on subsequent storage and therefore should be replaced with freshly resuspended material at three- or four-month intervals.

PROTECTION TESTS

Protection tests are made of a sample of each lot of concentrated serum. Concentrated sera may be diluted on the basis of their agglutinative and protective titers. For final standardization, protection tests are made on the diluted, filtered material shortly before it is to be dispensed for distribution. Different amounts of each serum diluted with a mucin preparation are mixed with the test dose of culture suspended in mucin and injected intraperitoneally into mice (7, 20, 21). In each test, as a basis of comparison in estimating the potency of the sera, mice are injected with mixtures of different amounts of the control serum and the test dose of culture. As a culture control, the virulence is tested by injecting mice with different doses of the mucin-culture suspension, selected to include the minimum lethal dose.

Cultures.—It is important to employ a highly-virulent, rapidly-growing, young culture. For the test, a transfer is made on glucose serum agar from a culture recently recovered from the heart's blood of a mouse or from a dried preparation and incubated at 34°-36°C. for from five to six hours under partial CO_2 tension.

At present, the following strains are used: group I, No. 69, group II, No. 39635, and group IIa, No. 3855. Additional strains are employed from time to time.

The strains are maintained in the dry state or passed through mice at intervals of one or two weeks and the cultures are recovered from the heart's blood. If dried cultures are used, the subcultures are transferred rapidly (daily or twice daily) at least three times before the test culture is inoculated.

From the test culture, make a slide preparation stained by Gram's method. Suspend the growth in 1 or 2 ml. of beef-infusion broth (*F2A*), filter through cotton (see p. 622), and dilute the suspension with broth so that it equals in density glass standard No. 3. Such a suspension contains approximately 2000 million microorganisms per milliliter.

Test Dose of Culture.—The test dose of culture is determined for each strain of meningococcus and is expressed as the concentration of the standardized culture suspension. It is selected on the basis of the results of protection tests in which different amounts of culture are used as test doses and should be, whenever possible, in the range where the ratio of the dose of culture to the protecting dose of serum is constant. Standardization tests with the optimum test dose should give uniform results in repeated tests.

The test dose of culture varies with the strain and the serologic group. At present, with strain No. 69, group I, a concentration of 10^{-2} of the standardized suspension is used. Since 0.5-ml. amounts of the selected culture dilution are combined with 0.5 ml. of the serum dilutions, the injected dose is approximately 10 million microorganisms; with groups II and IIa strains, the concentrations are usually 10^{-4} and 10^{-3} respectively, or approximately 100,000 and 1 million microorganisms.

Make the preliminary dilutions of the test culture in broth; final dilutions that are to be injected, in mucin suspensions.

Preparation of Mucin Suspensions.—Suspensions of granular mucin (Type 1701-W, Wilson Laboratories, Chicago, Illinois) are prepared in 500-ml. amounts. The concentration of mucin required may vary with the particular batch or with the test strain. Four- or five-per-cent suspensions have been employed; at present, four-per-cent is being used for groups I and IIa meningococcus cultures, and five-per-cent for group II. Modifications in the method may be necessary with different batches of mucin.

Procedure: For a four-per-cent suspension, to 20 grams of mucin in a 1-liter Erlenmeyer flask, add gradually, with frequent shaking, 400 ml. of distilled water. Allow the mixture to stand for from one-half to one hour. Rotate the flask vigorously several times during this period. To ensure a uniform suspension, transfer the material to a glass mixing jar and stir with an electric stirrer for from three to five minutes. Rinse the flask with 50 ml. of distilled water, add the rinsings to the suspension and stir again for two minutes. Pour the suspension into a fresh flask. Rinse the mixing jar with 50 ml. of distilled water and add the rinsings to the flask. By means of the spot-plate method, determine the amount of NaOH required to adjust the suspension to about pH 6.7 using bromothymol blue as indicator. Adjust to about pH 6.7 instead of pH 7.0 if bromothymol blue is used because of the protein error in the colorimetric method. For the titration, use 5 ml. of a 1:5 dilution of the mucin suspension and 0.05 *N* NaOH. Then calculate the amount of 1 *N* NaOH required for the total volume. After the addition of the latter, mix the suspension thoroughly. Check the pH reaction and if necessary adjust. Have the mucin suspension sterilized at 5 pounds pressure for fifteen minutes. After sterilization, streak a blood-agar plate and inoculate a tube each of aerobic and anaerobic "sterility-test" broth. Remove a small sample for determination of the final pH. Store the mucin preparation in the cold room. Discard it if the sterility tests indicate contamination. Each time part of a flask of the suspension is used, streak a blood-agar plate before storing the remainder.

The Test

Healthy white mice weighing between 16 and 20 grams are used. For final titrations of sera, use 10 mice for each serum dilution; for preliminary tests, 3 or 5 mice. The procedures for the test are the same as those for protection tests of antipneumococcus serum, p. 684, except as indicated.

Dilutions.—Serum: Dilute the test and the control sera in a mucin preparation so that the desired amounts are contained in 0.5 ml. Pipette the required amounts of mucin for the serum dilutions into 5- or 10-ml. cork-stoppered bottles.

Culture: Dilute the standardized culture suspension in serial 10-fold dilutions. Make intermediate dilutions with broth, final dilutions for injection with a mucin preparation so that the required test dose to be combined with the serum dilutions is contained in 0.5 ml. Dispense the test-dose dilution in stoppered containers, one for every 10 mice to be injected. For the culture controls prepare the dilutions so that the required amounts are contained in 1 ml. of mucin. Pipette the broth for the intermediate dilutions for the culture controls into the necessary number of tubes and the mucin for the final dilutions into 5-ml. bottles with cork stoppers.

Culture Control.—Make poured plates with one or two of the highest dilutions of culture prepared in mucin immediately after the dilutions are made and again after the completion of the test. Pipette into Petri plates 0.5-ml. amounts of the dilutions in duplicate, pour into each plate melted beef-infusion agar (*F35A*) to which defibrinated rabbit blood not more than one week old has been added immediately before to give approximately a 2-per-cent concentration. Mix well by a gentle rotary motion. Incubate at 34°–36°C. under partial CO_2 tension for seventy-two hours and count the colonies.

For the virulence control, inject groups of three mice with approximately 1, 10, and 100 M.L.D. respectively. These doses are contained in 1-ml. amounts of mucin, and the final concentrations are usually 5×10^{-9}, 5×10^{-8}, and 5×10^{-7} of the standardized culture suspension (about 10, 100, and 1000 microorganisms).

Injections. Observation of Animals. Necropsies.—All injections are made intraperitoneally and are completed within one hour from the time the culture is suspended. Use 2-ml. syringes and 22- or 23-gauge needles, one-half an inch long. Inject first the mice that receive mixtures of the control serum and culture and then the test sera mixtures. Inject last the mice for the virulence control.

Inspect mice four times daily for the first three days, at about 9:00 a.m., 1:00 p.m., 5:00 p.m., 11:00 p.m., and, when indicated, at 5:00 a.m. also. See p. 685.

When autopsies are to be made, perform them as soon as practicable. With the heart's blood, streak a blood-agar plate and make a slide preparation. Autopsy all mice that die unless more than sixty per cent in any one group dies; then autopsy at least two of three, three of five, or six of ten mice used for any one dose of culture or serum-culture mixture.

Interpretation of Test. Estimation of Potency.—Mice inoculated with serum-culture mixtures and surviving for ninety-six hours are considered protected by the amount of serum with which they were injected. The M.L.D. of the culture is considered to be the smallest number of microorganisms that will kill in ninety-six hours more than fifty per cent of a group of mice weighing from 16 to 20 grams. If contaminating microorganisms are isolated at autopsy and no meningococci are recovered in culture or seen in the blood film, the mouse is disregarded in the interpretation of the test. No test is considered satisfactory unless at least one dose of the test serum and one of the control serum protect for at least four days and unless the highest dilutions of the control and test serum fail to protect more than 50 per cent of the mice. The titer of a serum is based on the fifty-per-cent survival end point estimated according to the method of moving averages (see p. 686) and is compared with that of the control serum. The potency of final preparations of serum is based on similar results in two satisfactory tests. The titer of a lot of serum distributed for therapeutic use should equal or exceed that of the control serum.

PRECIPITATION TESTS

Preliminary precipitation tests are made of samples from trial bleedings and from large bleedings after the preservative is added. Varying dilutions of the sera to be tested and of the control serum are combined with equal volumes of appropriate dilutions of ultrafiltered residues of broth culture filtrates. The reactions are controlled by testing the antigens with univalent group sera and with normal horse serum. The mixtures are incubated at 34°–36°C. for two hours and placed in the cold room overnight.

Antigens.—The following standard strains are usually employed: group I, Nos. 69 and 68; group II, No. 38; group IIa, No. 3855. Cultures are grown for five days in veal-infusion broth (*F2A*) with 0.75-per-cent glucose dispensed in 400-ml. amounts in 2-liter Erlenmeyer flasks. The purity of the cultures is checked by examining slide preparations stained by Gram's method and by streaking blood-agar plates. Phenol is added to the cultures to give a final concentration of 0.5 per cent. The cultures are pooled, clarified by filtration through asbestos wool, and then passed through a Mandler filter candle. After the completion of sterility tests, the filtrate is ultrafiltered through a 4.5-per-cent nitrocellulose (Parlodion) membrane (22). Four liters of filtrate are usually concentrated to about 50 ml., see p. 815. The residue is adjusted to isotonicity with a 17-per-cent sodium chloride solution. Sterility tests are made and if a residue is found to be contaminated, it is filtered at once.

The Test

The procedures are the same as those described in "General Bacteriologic Technic," p. 21, except as indicated.

Dilutions.—It is essential to use absolutely clear reagents. Therefore, if either the test sera or the antigens contain suspended material, centrifuge them before proceeding with the dilutions, which are made in salt solution.

Serum: From 4 to 6 dilutions, usually in geometric progression with increments of 100 per cent, are tested. The range depends upon the potency of the sera. Generally, dilutions of from 1:5 to 1:160 or higher are made. The general procedure for the preparation of the dilutions is that described under "General Bacteriologic Technic," p. 24.

Ultrafiltered residues: From the stock solution prepare the required dilution of each ultrafiltered residue for the test. To determine the optimum dilution of a residue, make a preliminary test of several different dilutions each combined with a series of 3 or 4 dilutions of the control and univalent group sera. Note the immediate ring reactions as well as those after incubation for two hours and overnight storage in the cold room. Select as the optimum, the dilution that gives the strongest reactions and the most clear-cut particulation with the control and the homologous group serum with little or no cross reactions with heterologous group sera. At present, dilutions of 1:15 or 1:20 are used.

Mixtures.—Use unplugged clear tubes (100 by 8 mm.). Add 0.1-ml. amounts of the diluted antigens to 0.1 ml. of each of the serum dilutions. Shake the racks vigorously and make readings after incubation for two hours and overnight storage in the cold room.

Reading and Recording Results.—Read and record the degree of reaction as described on p. 22. The titer of the serum is the maximum final dilution in which precipitation definitely visible to the unaided eye occurs. The titer of the serum selected for concentration should equal or exceed that of the control serum.

AGGLUTINATION TESTS

Agglutination tests are made of samples of sera from the horses under immunization from time to time, and from each concentrated and final preparation. Varying dilutions of the sera to be tested and of the control serum are mixed with equal volumes of salt-solution suspensions of the strains used in immunization. The reactions are controlled by mixing the suspensions with univalent group sera, normal horse serum, and salt solution. Occasional tests are also made with additional strains, including freshly isolated cultures, atypical as well as those classified as groups I, II, and IIa.

The Test

The procedures are the same as those described in "General Bacteriologic Technic," p. 19, except as indicated.

Serum Dilutions.—From six to nine dilutions of serum are usually tested, ranging from 1:100 to 1:7500. The normal horse serum used as a control is diluted 1:100 and 1:250; the univalent rabbit sera are so diluted that characteristic reactions are obtained with their homologous strains and the occurrence and degree of cross agglutination are indicated (generally 1:100 and 1:400). For the production of the univalent sera, see "Production and Standardization of Sera and Other Preparations for Diagnostic Use," p. 785.

Set up the necessary number of tubes (150 by 19 mm.). Record the serum number or horse number, date of bleeding, and serum dilution on the first tube of each series, the serum dilution on the other tubes, and on every tube the required volume of salt solution. Add the salt solution to all tubes from a burette. With a 1-ml. pipette, add the serum, 0.5 ml. for the first dilution of horse serum, 0.1 ml. for rabbit serum, to the first tube of the series. With a fresh 2-ml. pipette, mix the dilution thoroughly by drawing up and discharging several times, and transfer the required volumes for the rest of the dilutions.

Bacterial Suspensions.—Prepare the suspensions immediately before they are to be added to the serum dilutions. Use 16- to 18-hour cultures grown on glucose serum agar. Dilute the suspensions with salt solution so that all are of a uniform turbidity and equal in density to glass standard No. 3. Shake the standard suspension well just before using. Mix the bacterial suspensions thoroughly by rotating the tubes.

Serum-Culture Mixtures.—After preparing the serum-culture mixtures, flame the mouths of the tubes, then shake the racks well and place them in the incubator at 55°C. for twenty-one hours. Shake the racks again and return them to the incubator for three hours before making readings.

Reading and Recording Results.—Read the reactions by artificial light against a dark background. Examine mixtures before and after shaking. Record the degree of reactions as described on p. 21.

The titer of the serum is the maximum final dilution in which clumping definitely visible to the unaided eye (+ reaction) occurs. Should irregularities occur in the reactions with a culture, the test with that culture is considered indeterminate. The titer of the serum distributed for therapeutic use should equal or exceed that of the control serum.

PREPARATION FOR DISTRIBUTION

As soon as the sterility and standardization tests are satisfactorily completed, the serum may be bottled for distribution. Sera for therapeutic use are dispensed in 20-ml. amounts. If kept under proper conditions, the serum is considered satisfactory for use for eighteen months from the date of the last satisfactory test. In each package of serum is included a vial containing 1 ml. of a 1:10 dilution of normal horse serum for use in sensitivity tests, see p. 689.

The serum for diagnostic use is dispensed in 5-ml. volumes. High-titered unconcentrated serum of broad valency is selected on the basis of agglutination tests with recently isolated as well as the standard strains.

CLASSIFICATION OF MENINGOCOCCUS STRAINS

The serologic classification of meningococcus strains isolated from patients treated with the serum produced in this laboratory, and frequently strains from other cases, is determined. The cultures are classified on the basis of their agglutination, precipitation, and capsular swelling reactions with univalent group sera. For production of the univalent group sera in rabbits, see "Production and Standardization of Sera and Other Preparations for Diagnostic Use," p. 785. Univalent sera produced in horses are used in the precipitation tests on immune-serum-agar plates. Strains are classified as group I, group II, group IIa (encapsulated (12, 14)), or group "X" (atypical (23)). A preliminary slide agglutination and a confirmatory plate precipitation test is made of each strain. If the results of these tests fail to indicate the group relationship, a tube agglutination test is made. In the case of group-IIa strains or others of special interest, a capsular-swelling test is also made.

Because of the convergence in serologic reactions of the standard strains originally classified as group I and group III, as well as the frequent isolation of strains that could not be classified except as group "I or III" on the basis of their agglutinative activity, and the failure of precipitation tests to differentiate between these two groups, the designation "I–III" was for a time used for all strains related to either or both. To simplify the nomenclature, group I is used to connote both groups I and III.

Identification and Purity Tests.—Promptly on receipt of a culture, make a slide preparation stained by Gram's method. Inoculate a glucose serum agar slant and one tube of ascitic-fluid semisolid medium (*F36A*), streak a blood-agar plate, and incubate the cultures at 34°–36°C. under partial carbon dioxide tension. See "General Bacteriologic Technic," p. 11. Occasionally, cultures that fail to grow on blood or glucose serum agar develop in the semisolid medium. If after from eighteen to twenty-four hours the growth is good and the morphologic and cultural characters are typical, test the glucose serum agar culture for fermentative activity. For procedures followed in maintenance and storage of strains and in fermentation tests, see p. 698. Determine the serologic reactions by means of slide agglutination and plate precipitation tests, and, when indicated, by tube agglutination and capsular-swelling tests.

AGGLUTINATION TESTS

The strains are tested for agglutination reactions in both the macroscopic slide and tube tests with univalent rabbit sera produced with the standard strains group I, No. 68, group II, No. 38, and group IIa, No. 3855, and in the tube test, also with multivalent horse serum. In addition, sera produced with other strains, freshly isolated and stock of the different groups, and atypical strains including group "IV", are occasionally used in special tests.

Slide Agglutination.—For technic, see p. 21. Use the sera undiluted or diluted 1:10. Place a loopful of each of the three group sera on a glass slide. The test culture grown on glucose serum or blood agar should not be more than twenty-four hours old.

Tube Agglutination.—The sera are usually diluted 1:25, 1:50, 1:100, 1:200, and 1:400. The technic is the same as that described on p. 706.

except that the test is incubated at 34°–36°C. for two hours and then left in the cold room overnight.

Interpretation of Results.—Strains are classified as group I, II, or IIa on the basis of the results of the slide agglutination test if they are agglutinated strongly by the serum of one group with little or no cross agglutination in the sera of the heterologous groups; on the basis of the tube agglutination tests, if they are agglutinated by the serum of one group in a final dilution of 1:100 or higher with slight or no cross agglutination in the heterologous sera, or if they are agglutinated in final dilutions of 1:200 or higher with relatively little cross agglutination.

IMMUNE-SERUM-AGAR-PLATE PRECIPITATION TESTS

For the precipitation tests, groups I, II, and IIa and multivalent sera produced in horses are used. Plates are prepared with beef-infusion agar to which 5 per cent of immune serum has been added. After inoculation the plates are incubated and the reactions appearing as haloes around the area of growth are recorded (6, 24).

Preparation and Inoculation of Plates.—Use 1.75-per-cent beef-infusion agar containing 0.2-per-cent glucose (*F35A*) dispensed in 15-ml. amounts. Melt the agar, cool to about 42°C., and add 0.75 ml. of the proper immune serum to each tube. Mix the contents thoroughly by a rotary motion, and pour immediately into sterile Petri plates. Incubate the plates at 34°–36°C. for from eighteen to twenty-four hours. If the immune-serum agar appears to be sterile, store the plates in the cold room until required.

To inoculate, implant with a loop on the surface of the agar small dabs of from 18- to 24-hour cultures grown on glucose serum agar. Include, as a control in each test, cultures of standard groups I, II, and IIa strains. Incubate the plates at 34°–36°C.

Reading and Interpretation of Results.—After twenty-four, forty-eight, and seventy-two or ninety-six hours, read the plates by artificial light against a dark background. Record the varying degrees of reactions as 4+, 3+, 2+, + (slight but definite precipitation), tr. (trace or very delicate halo), r. (ring, clear zone adjacent to the area of growth with sharply defined ring of precipitation beyond), and − (no reaction). Two zones of precipitation, the inner one the denser, are frequently noted. Record also the diameter of the area of growth and the reaction in millimeters.

For example:

3	(diameter of area of growth)
14(4+)	(diameter of area of reaction and growth and degree of reaction of inner zone)
18(tr.)	(total diameter of area of reaction and growth and degree of reaction of outer zone)

Strains that show definite reactions with group-I or group-IIa and multivalent sera and little or no cross reaction with heterologous sera are classified as group I or group IIa; those that react with group-II and multivalent sera and not with the heterologous sera, as group "X" related to group II. Group-II strains, in general, exhibit only slight precipitative activity. Thus, a strain cannot be classified as group II unless the agglutination reactions are distinct. Strains that cannot be classified by either the agglutination or the precipitation reactions are included in group "X" (atypical).

CAPSULAR-SWELLING TESTS

For the capsular-swelling tests, groups I and IIa undiluted sera produced in rabbits are used (12, 25). Group-II serum that lacks capsular-swelling activity is occasionally included as a control. Standard strains of groups I and IIa are included in every test. The control and test cultures are grown in glucose serum agar for from five to six hours under partial CO_2 tension, suspended in broth and diluted to equal in density glass standard No. 3. A further 1:2 dilution in broth has been found to give a suspension of suitable concentration.

Mixtures.—Use three loops for preparing the mixture of culture suspension, serum, and alkaline methylene blue stain: one with inside diameter of about 1 mm. for the culture, one of 5 mm. for the serum, and one of 3 mm. for the stain. Mix one loopful each of the culture, serum, and stain on a glass slide. Place a cover glass over the mixture and seal the edges of the cover glass with vaseline. Keep the slides at room temperature for from twenty to thirty minutes.

Reading and Interpretation of Results.—Use a microscope lamp that provides intense illumination and examine the mixtures with an oil-immersion objective. Adjust the iris of the microscope condenser to give maximum definition. Record the degree of reaction as 3+ (maximum capsular swelling), 2+ (clear-cut capsular swelling but narrower than 3+), + (discernible but slight reaction), ± (doubtful reaction). Strains of groups I and IIa, with rare exceptions, exhibit definite capsular swelling. While the degree of the specific reaction may vary, cross reactions with the heterologous serum have not been noted. Thus, the capsular-swelling reaction of a strain with one or the other test serum clearly indicates the group relationship.

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CHAPTER 55

PURIFICATION AND CONCENTRATION OF PLASMAS AND SERA OF IMMUNIZED HORSES AND RABBITS

PURIFICATION AND CONCENTRATION OF ANTITOXIC PLASMAS

DIPHTHERIA, TETANUS, BOTULINUS, STREPTOCOCCUS

Fractionation with Ammonium Sulfate

Diphtheria, tetanus, botulinus, and streptococcus antitoxic plasmas are purified and concentrated as a routine procedure. The method of concentration is based on the fact that the antitoxin is associated chiefly with the pseudoglobulin fraction of the protein. The euglobulin is precipitated by adding saturated ammonium sulfate solution to the plasma to a concentration of 29 per cent and heating. The precipitate is then removed by filtration, and the pseudoglobulin with its associated antitoxin is precipitated by increasing the content of saturated ammonium sulfate solution in the filtrate to 48 per cent. Excess moisture and some ammonium sulfate are removed from the precipitate by pressure, and the remaining ammonium salt by dialysis. Further purification is accomplished by isoelectric precipitation (1) of the residual euglobulin, after which the pseudoglobulin is reprecipitated with ammonium sulfate. This precipitate is pressed and the ammonium salts removed by dialysis. Sodium chloride to give a concentration of 0.8 per cent and preservative are added to the dialyzed material, and the hydrogen-ion concentration is adjusted to that of the blood.

Preliminary Treatment of Plasma

When plasma is received, sufficient 90-per-cent phenol is generally added with constant stirring to give a concentration of 0.3 per cent and the material is stored in the cold room until concentration is commenced. One lot usually consists of approximately 60 liters, which represent about 12 bleedings.

Pooling of Plasma.—Decant the supernatant from each bleeding and record the volume. Measure the amount of residue separately and add this figure to the previous one to obtain the total. Pool the supernatants. Centrifuge the residues and add the clear portion to the pooled material. Mix thoroughly by stirring.

Adjustment of the Hydrogen-Ion Concentration: When the plasma contains phenoloid preservative, its reaction is adjusted colorimetrically to pH 8.0 which is equivalent to the same value electrometrically. In the absence of phenoloid preservative, there is a large protein error of the indicator which requires that the adjustment be made to a colorimetric value of pH 7.3 to approximate pH 8.0 obtained by the electrometric method.

Pipette 10 ml. of pooled plasma into each of two tubes (150 by 15 mm.) and to one tube add 0.4 ml. of 0.02-per-cent phenol red indicator. From a burette add 0.1 N ammonium hydroxide (NH_4OH) drop by drop to the tube of plasma and indicator. Shake well after each addition and compare frequently with the buffer standard (pH 8.0) until the same shade of color is obtained. From the volume of 0.1 N ammonium hydroxide used to adjust the plasma in the tube, calculate the volume of N ammonium hydroxide necessary to change the whole volume of plasma as follows:

$$\text{Vol. of } N\ NH_4OH = \frac{\text{Vol. of plasma}}{10} \times \frac{\text{Vol. of } 0.1N\ NH_4OH \text{ used}}{10}$$

Stirring constantly, add slowly to the plasma the calculated amount of N ammonium hydroxide and redetermine the pH value. Should the adjusted plasma be too acid, determine the additional volume of N ammonium hydroxide required by titrating with 0.1 N ammonium hydroxide as described. If too alkaline, titrate with 0.1 N hydrochloric acid. Make any final adjustments required to give pH 8.0.

First Precipitation and Filtration

The euglobulin and fibrinogen of the plasma are precipitated by adding saturated ammonium sulfate, $(NH_4)_2SO_4$, solution to give a concentration of 29 per cent and heating to 60°C., which increases the precipitation of nonspecific protein.

Special supplies and apparatus. Bottles: Approximately sixty 8-liter flint-glass bottles, half of them graduated at 5500 ml.

Water bath: A specially designed insulated copper water bath with inside dimensions of 5 by 2½ feet with a depth of 29 inches. The bottles rest on perforated trays raised 3 inches from the bottom, permitting free circulation of water. The bath is filled and emptied by permanent water connections and heated by either steam or gas.

Saturated ammonium sulfate solution: To prepare the solution place 100 pounds of powdered ammonium sulfate (technical grade) in a 110-liter glass-lined steel container. Add approximately 50 liters of tap water heated from 70° to 80°C. Stir continuously for twenty minutes, using an electric stirrer with a wooden or stainless steel paddle. Cool to room temperature. Determine the specific gravity with a hydrometer; if below 1.245, add ammonium sulfate and stir. Redetermine the specific gravity. Filter the saturated solution through soft filter paper into properly labeled bottles for future use. After several solutions have been made there will be a considerable residue left in the container, each liter of which may be considered as equivalent to a pound of dry salt in the preparation of new solutions.

Addition of Ammonium Sulfate.—Distribute the adjusted plasma in 3-liter amounts in 8-liter bottles. Dilute the plasma with half its volume of tap water, add the required volume of saturated ammonium sulfate solution, calculated according to the following formula:

$$X = \frac{CV}{100 - C}$$

X = volume of saturated $(NH_4)_2SO_4$ solution
C = desired concentration in per cent by volume of saturated $(NH_4)_2SO_4$ solution
V = volume of diluted plasma

Heating of Salted Plasma.—Place the bottles of plasma in the bath and fill it with water to the level of the material in the bottles. Bring the temperature of the water rapidly to 60°C. and hold it at this point until the temperature of the plasma reaches 58°C., usually in about two hours. Raise the temperature of the water to from 61° to 61.5°C. and bring the plasma to 60°C. in one hour. Hold at this temperature for thirty minutes. The temperature of the water should never be above 62°C. Stir the plasma and the water every fifteen minutes during heating. Cool the plasma gradually to room temperature by allowing a slow stream of cold water to enter at the bottom of the bath and escape through an overflow pipe near the top.

Filtration.—By filtering through soft filter paper, the euglobulin, fibrinogen, and other proteins contained in the precipitates are separated from the pseudoglobulin and albumin held in solution.

Special supplies and apparatus. Funnels: Heavy ribbed funnels, 21.5 cm. outside diameter.

Filter paper: Soft filter paper, 450 mm. in diameter. Eaton and Dykeman Co. No. 615.

Filter racks: The filter racks are constructed on the principle of a bottled-water stand. They consist of a removable top shelf, a second shelf 6 inches lower, and a third 22 inches below the second. Circular openings in the top shelf, 6½ inches in diameter, are directly above openings in the second, 3½ inches in diameter. The funnels are protected in front by hinged doors. (See Fig. 64.)

Place fluted filter paper in the funnels in the racks and a graduated 8-liter bottle under each funnel. Invert the full bottles over the funnels until they rest on the rim of the upper openings. Filtration should now proceed automatically. It is usually convenient to commence filtration in the afternoon and to allow the filters to remain in place until the following morning.

Washing of Precipitate.—When filtration is completed, wash the precipitate to remove any adhering pseudoglobulin. Transfer the filter papers

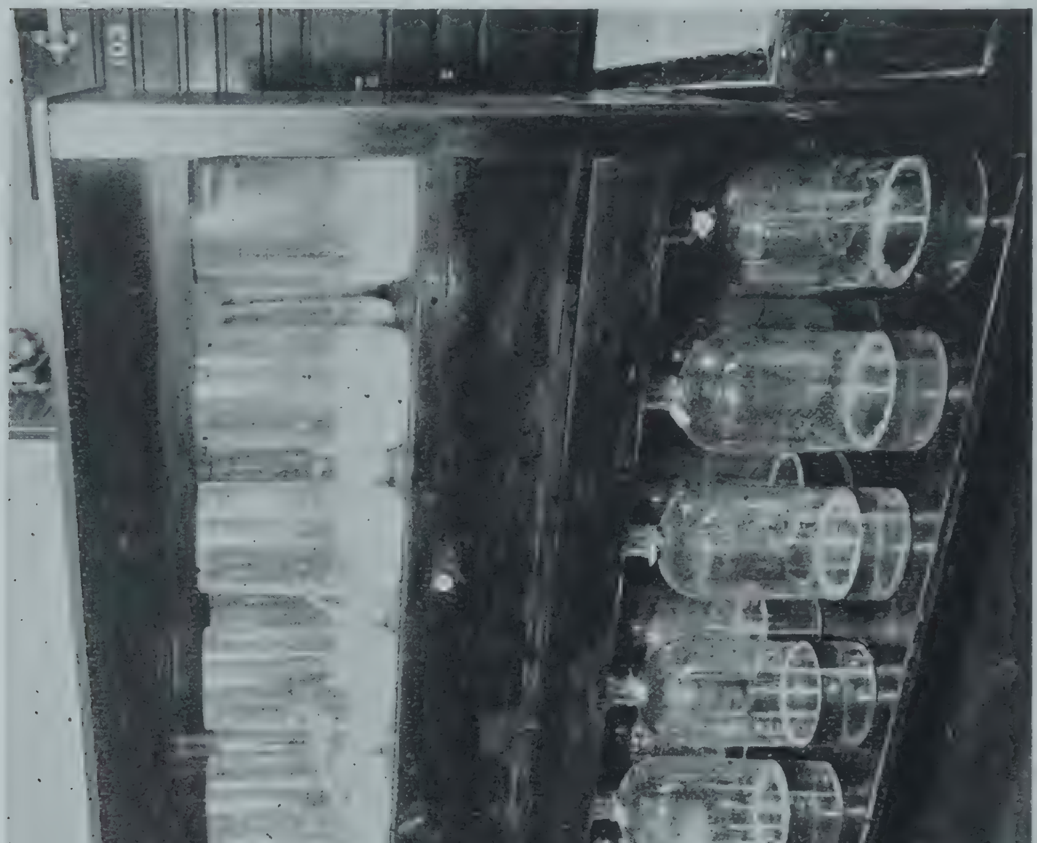

Fig. 64. Filter Racks

and contents from every 2 funnels to a container and add approximately 2 liters of 31-per-cent saturated ammonium sulfate solution (2 per cent higher than that used for precipitation). Macerate the paper thoroughly with a heavy glass rod and filter the material through fresh filter papers into the original bottles of filtrate. When drained, discard the precipitate.

Second Precipitation and Filtration

The pseudoglobulin is precipitated from the filtrate by increasing the concentration of saturated ammonium sulfate solution to 48 per cent and is separated by filtration from the albumin, which remains in solution.

The following formula is used to calculate the required volume of ammonium sulfate, concentrations being expressed as per cent by volume of saturated ammonium sulfate solution:

$$X = \frac{V(C_2 - C_1)}{100 - C_2}$$

X = volume of saturated ammonium sulfate to give the required concentration
V = volume of initial solution
C_1 = initial concentration of ammonium sulfate
C_2 = desired or final concentration of ammonium sulfate

Dispense the first filtrate in measured volumes, not exceeding 5500 ml., in 8-liter bottles. Add to each the required volume of saturated ammonium sulfate solution and mix thoroughly. Allow the mixtures to stand for one hour. Mix the contents of each bottle, then distribute the material among sufficient bottles so that each contains from 4500 to 5000 ml. Filter, as previously described, through hard filter paper (Whatman No. 50). Five days are usually required for the precipitates to drain thoroughly.

Drying of Second Precipitate

The excess moisture in the precipitate as well as some of the ammonium sulfate is removed by means of absorbent paper and pressure.

Special supplies and apparatus. Absorbent papers: Soft filter paper, 50 by 50 cm. With care the paper may be used repeatedly.

Muslin cloths: Heavy muslin cloths, 50 by 50 cm., with hemmed edges.

Press: Cider press No. 4, manufactured by the Ames Plow Co., Boston, Massachusetts.

Drier for absorbent papers: At present a steam laundry drier is used.

Preparation for Pressing.—When the precipitates are drained, remove each filter paper with its contents and lay it on a pad of from 50 to 75 sheets of absorbent paper. Divide the precipitate from every third paper between the preceding two. With a hard rubber spatula work the precipitates into flat disks. Re-work after about one hour and again if necessary.

Pressing. When the material has dried to the consistency of vaseline, scrape each precipitate toward the center of the paper, and place the precipitates face to face in pairs. Place the precipitates in the press between pads of about 75 sheets of absorbent paper. Use care to keep the stack level. If necessary add but 5 pairs at a time and apply moderate pressure after each addition. Tighten the press from time to time during the first day and leave the precipitates in the press overnight. If the material is to be purified further by isoelectric precipitation, discontinue the pressing at this point. The following morning, to prevent breaking the papers as the pressure is increased, replace the hard filter papers with muslin cloths. Carefully peel off the top paper, disturbing the caked precipitate as little as possible. Place a single layer of muslin beside the precipitate. Invert the precipitate onto the muslin cloth. Peel off the second paper. Remove any precipitate adhering to the papers, break up the cake, and cover with a second cloth. Return the precipitates between fresh pads to the press, using the same care as before to keep the pile level. Increase the pressure gradually and leave the precipitates in the press overnight or until practically no more moisture is absorbed. The material is then ready for dialysis.

Removal of Ammonium Sulfate by Dialysis

The pseudoglobulin and antitoxin are freed from the ammonium salts by dialysis. Dialysis should be continued until not more than 0.2 per cent of salt remains, usually for five days.

Special supplies and apparatus. Cellophane for dialyzing bags: No. 600 purchased from the E. I. duPont de Nemours and Co., Cellophane Division, Wilmington, Delaware, in rolls 24 or 30 inches wide, and cut in circular sheets approximately 24 or 30 inches in diameter.

Dialyzing bath: Alberene baths, with drain boards, installed in the dialyzing room which is kept at approximately 5°C., are supplied with running tap water also cooled to the same temperature. Removable cross bars of galvanized iron piping are placed parallel to the long sides of the bath. (See Fig. 65.)

Preparation for Dialysis.—Lay a sheet of Cellophane on a flat surface. Remove the upper muslin cloth from one of the precipitates. Loosen and scrape the precipitate onto the cellophane sheet. Gather the edges of the sheet together to form a bag and tie.

Dialysis.—Adjust the stream of water to give a slow even flow. Tie the bags to the cross bar so that they are one-third submerged and dialyze for five days. Use separate cross bars for each lot of material.

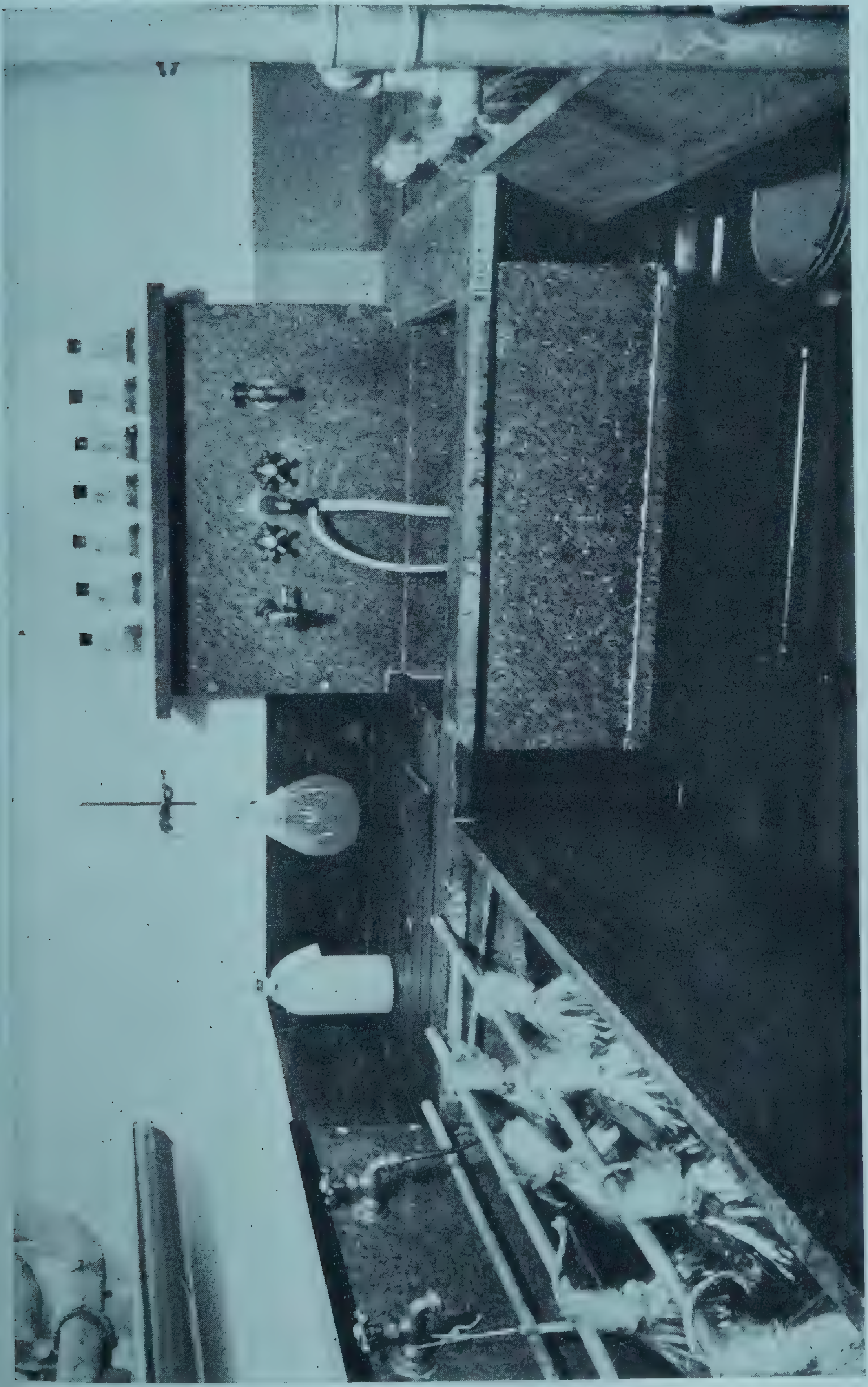

Fig. 65. Refrigerated Dialyzing Room

While tests have shown that five days are sufficient to remove practically all of the ammonium sulfate, it is advisable occasionally to ascertain the efficiency of dialysis by determining the percentage of ammonium salts in the dialyzed material.

Pipette 1 ml. of the dialyzed antitoxin into a 300-ml. Erlenmeyer flask and add 20 ml. of 2.5-per-cent trichloracetic acid. To prevent excessive evaporation during heating, stopper the flask with a 1-holed rubber stopper through which a piece of glass tubing about 60 cm. long is passed. Heat in a bath of boiling water for five minutes and cool rapidly. Filter and wash the precipitate with ammonia-free water. Dilute the filtrate to bring the comparative readings with Nessler standards within the range of greatest accuracy, as in the following table.

Approximate content of $(NH_4)_2SO_4$ %	Final volume *ml.*
0.05	100
0.10	200
0.20	400
0.30	500

Transfer 10 ml. of the diluted filtrate to a 50-ml. Nessler tube and make up to the mark with ammonia-free water. Add 1 ml. of Nessler reagent, allow the mixture to stand for ten minutes, and compare with the standards. (The standards and reagent are those prepared for water analysis (2).) Calculate the percentage of ammonium sulfate in the original sample, as follows:

Assuming that the reading is 0.00001 gram of nitrogen and that the original sample was diluted to 200 ml., then:

$$0.00001 \times \frac{200}{10} \times 100 \times \frac{132.2}{28} = 0.094 \text{ per cent } (NH_4)_2SO_4$$

where 132.2 and 28 are respectively the molecular weights of $(NH_4)_2SO_4$ and N_2.

Further Purification of the Antitoxin by Isoelectric Precipitation

Antitoxin concentrated by ammonium sulfate fractionation is usually purified further by the removal of the residual water-insoluble globulin by isoelectric precipitation. This method is used to increase the potency concentration of the antitoxin, to improve its appearance, and to eliminate to a large extent precipitation on standing.

Isoelectric Precipitation.—Pool the dialyzed material and determine the volume. Rinse the dialyzing bags with a portion of the water to be used for dilution. Dilute the material to the original volume of the plasma with distilled water containing 0.3 per cent of cresol. Pipette 10 ml. of this dilution into each of 5 tubes (150 by 15 mm.). Add to 1 tube 0.1 ml. of 0.05 *N* hydrochloric acid and to the others volumes increasing by 0.1 ml. progressively. Separate the precipitates by centrifugation. To the clear supernatant from the first tube, add 1 drop of 0.05 *N* hydrochloric acid. If a precipitate forms, test similarly the supernatant from the second tube. Continue until a tube is reached in which the addition of the drop of acid produces no precipitation.

Calculate the amount of N hydrochloric acid required to adjust the main volume of material according to the following formula:

$$\text{ml. } N \text{ HCL required} = \frac{\text{Vol. of material}}{10} \times \frac{\text{Vol. } 0.05N \text{ HCL used}}{20}$$

Add the calculated amount of N hydrochloric acid and mix thoroughly. Pipette 10 ml. of the adjusted mixture into each of 2 tubes and remove the precipitate by centrifugation. To the clear supernatant from 1 tube add a drop of 0.05 N hydrochloric acid and to the other a drop of 0.05 N sodium hydroxide. No precipitate should form in either tube. If it does, further adjustment is necessary.

When the adjustment is completed, allow the material to stand overnight. In the morning siphon off the supernatant, centrifuge the residue, and add the second supernatant to the first. Distribute the material in 3-liter amounts in 8-liter bottles and add an equal volume of saturated ammonium sulfate solution to each. Proceed as in "Second Precipitation and Filtration," and the following sections.

Neutralization of Antitoxin and Addition of Preservative

The concentrated antitoxin is adjusted to pH 7.4 by the addition of sodium carbonate. Cresol is added as a preservative to give a final concentration of 0.3 per cent. In order to reduce the precipitation of protein by the cresol, the sodium carbonate solution and the cresol are mixed before they are added to the antitoxin.

Special supplies and apparatus. Sodium carbonate, $Na_2CO_3 \cdot H_2O$, C.P.: Dissolved in sufficient water to form an 18.5-per-cent solution.

Container for pooling: Glass-lined steel container, 20-liter capacity.

Pool the antitoxin from the dialyzing bags and measure the volume. Add sufficient sodium chloride to give an 0.8-per-cent concentration. To determine the volume of 18.5-per-cent solution of sodium carbonate required to adjust the reaction to pH 7.4, follow the directions given under "Adjustment of Hydrogen-Ion Concentration," p. 713, with the following changes.

1. Adjust the antitoxin to 7.3 colorimetrically, which is approximately equal to 7.4 electrometrically.
2. Use for a sample 2 ml. of antitoxin diluted with 8 ml. of sodium chloride solution.
3. Use for titration a solution obtained by diluting 5 ml. of 18.5-per-cent sodium carbonate to 100 ml. in place of 0.1 N ammonium hydroxide.
4. Substitute 18.5-per-cent sodium carbonate for N ammonium hydroxide.
5. Use the formula:

$$\text{Vol. of 18.5-per-cent } Na_2CO_3 = \frac{\text{Vol. diluted } Na_2CO_3}{20} \times \frac{\text{Vol. antitoxin}}{2}$$

Calculate the amount of cresol and emulsify it with the sodium carbonate in a separatory funnel. Add the emulsion in a small stream to the antitoxin, stirring constantly. Rinse the funnel with 5 ml. of distilled water and add while stirring. Determine the pH of the adjusted material. Pour the material into sterile bottles.

The dialyzing bags, bottles, measuring jars, and other containers used in handling concentrated material are rinsed with distilled water and the rinsings pooled and stored in the cold room. At present, 'Merthiolate' to give a concentration of 1:10,000 is added as a preservative. After sufficient material (usually from 40 to 60 liters) has accumulated, it is pooled and reconcentrated by the procedure used for plasma except that 33 per cent of saturated ammonium sulfate is used for the first precipitation and this precipitate is not washed.

Adjustment of Total Solids Content

After a storage period of approximately one month, determine the total solids content and if it is above 20 per cent adjust.

Determination of Total Solids.—Carry out the determination in duplicate. Pipette 1 ml. of the solution to be tested into a weighed platinum dish. Dry to a constant weight in an oven at a temperature of from 103° to 105°C. The difference between the weight of the dish and that of the dish plus the residue, multiplied by 100 gives the per cent of total solids.

If the total solids content is above 20 per cent, the antitoxin must be diluted (usually to 18.5 per cent) with distilled water containing 0.8 per cent of sodium chloride and 0.3 per cent of cresol.

Formula:

$$\text{Final volume} = \frac{\text{Initial volume} \times \text{per-cent total solids}}{18.5}$$

After dilution, redetermine the total solids content by the same technic as in the first determination. Store the material in the cold room until required for filtration.

Determination of "Unit Concentration" and Loss in Total Number of Units

The efficiency of the process of concentration is followed by frequent determinations of "unit concentration" (increase in titer due to concentration of the antitoxic plasma) and loss in total units. To determine the "unit concentration," the units per milliliter of the concentrated material are divided by the units per milliliter of the plasma. To determine the loss in total units during the process of concentration, the total number of units in the concentrated material is subtracted from the total number in the plasma.

Digestion with Pepsin

This method of purification and concentration of the antitoxic plasmas is based on the procedure described by Pope (3). The plasma is diluted with water, the reaction adjusted to pH 3.8, and subjected to digestion with pepsin. The heat-labile globulins split off by the action of the enzyme are precipitated by heating to 58°C. in the presence of ammonium sulfate. The precipitate is removed by filtration and the antitoxic globulin in the filtrate is precipitated by the further addition of ammonium sulfate. This precipitate is filtered off, and after a short period of dialysis to remove most of the ammonium sulfate, further purification is accomplished by alum treatment to remove some of the nonspecific protein. The purified globulin is again precipitated with ammonium sulfate, the precipitate filtered off, pressed to remove excess moisture and some ammonium sulfate, and then dialyzed to remove the remaining ammonium sulfate. Sodium chloride to give a concentration of 0.8 per cent and preservative are added to the dialyzed material, and the hydrogen-ion concentration is adjusted to that of the blood.

Preliminary Treatment of Plasma

See section under "Fractionation with Ammonium Sulfate," p. 713. One lot consists of not over 40 liters, which represent about 8 bleedings for plasma.

Pooling of Plasma.—Pour each bleeding into a graduated precipitating jar and record the volume. Pool the plasma in a 10-gallon glass-lined container. Mix thoroughly by stirring with a rubber-tipped glass rod. Dilute the plasma with twice its volume of tap water. Divide the calculated volume of water between two 75-liter glass-lined containers and add one-half the pooled plasma to each container. Mix with a mechanical stirrer.

When antitoxin that has previously been concentrated by ammonium sulfate fractionation is to be treated, the dilution with water is increased to five times the volume because of the higher protein content of this material.

Digestion with Pepsin

Adjust the hydrogen-ion concentration of the diluted plasma to from pH 3.8 to 4.0. With constant mechanical stirring of the mixture, add slowly successive amounts of 2 *N* HCl. Determine the pH between each addition by means of the glass electrode (Beckman pH Meter). When the desired pH has been reached, add 0.25 per cent of pepsin (NF 1:3000) and 0.2 per cent of toluene. Continue stirring until the pepsin is dissolved. Allow the digestion to proceed at room temperature for from 20 to 22 hours.

Precipitation of the Heat Labile Globulins

Addition of Ammonium Sulfate and Adjustment of Reaction.—After digestion, add 150 grams of dry ammonium sulfate for each liter of the digested material and dissolve the salt by mechanical stirring. Continue the stirring and adjust the reaction to pH 4.6 by successive additions of 2 *N* NaOH. Determine the pH between each addition by means of the glass electrode until the desired value is obtained.

Heating.—After adjustment of the reaction, distribute the material in approximately 7-liter amounts in 8-liter bottles. Place the bottles in the bath and fill it with water to the liquid level inside the bottles. Bring the temperature of the water rapidly to 63°C. and hold it between 62° and 63°C. until the temperature inside the bottles reaches 50°C. Allow the bath to cool to 60°C. and hold the temperature at this point until the temperature inside the bottles reaches 57°C. Allow the bath to cool to 59°C. and keep it at this point until the temperature of the antitoxin is 58°C. Hold at this temperature for thirty minutes. During the heating, stir the material in the bottles frequently with a rubber-tipped, glass stirring rod. Cool the material gradually to room temperature.

Filtration and Washing of the Precipitate.—Filter through soft filter paper and wash the precipitate with a solution made by adding 160 grams of ammonium sulfate to each liter of water, see p. 715.

Precipitation of the Antitoxic Globulin. Filtration

Measure the filtrate from the first precipitate in a graduated glass precipitating jar and distribute it equally between two 75-liter glass-lined containers. Attach a mechanical stirrer to one of the containers and neutralize the solution by successive additions of 2 *N* NaOH. Determine the end-point, approximately pH 7.2, by the spot-plate method using phenol red indicator. After neutralization, add 200 grams of dry ammonium sulfate for each liter of solution. Stir until the salt is dissolved. Repeat this procedure with the second container. Distribute the precipitated material in approximately 7-liter amounts in 8-liter bottles. Allow to stand one hour. Filter through hard filter paper (Whatman No. 50). Two days are usually required for the precipitates to drain thoroughly.

Dialysis. Alum Treatment

Transfer the precipitate from 2 or 3 funnels to a circular sheet of Cellophane 30 inches in diameter. Dialyze for three days against cold running tap water. Measure the volume of the dialyzed material and pool it in a glass-lined container. Dilute with water to one-half the volume to which the original plasma was diluted. Stir mechanically and add 80 ml. of 10-per-cent aluminum potassium sulfate solution ($AlK(SO_4)_2 \cdot 12H_2O$) for each liter of diluted material. Continue stirring, and from a separatory funnel run in slowly 2 *N* NaOH until the pH value is between 7.6 and 7.8 as determined by the glass electrode. Stir for one hour and allow to stand two hours. Filter through soft filter paper.

Precipitation of the Antitoxic Globulin. Final Treatment

Distribute the filtrate following treatment with alum in 2500-ml. amounts in 8-liter bottles and add to each an equal volume of saturated ammonium sulfate solution. Proceed as in "Second Precipitation and Filtration" and the following sections, pp. 717 to 723, omitting "Further Purification of the Antitoxin by Isoelectric Precipitation," p. 720.

PURIFICATION AND CONCENTRATION OF ANTIPNEUMOCOCCUS AND ANTIMENINGOCOCCUS HORSE SERA

The specific activities of these sera are associated with the water-insoluble-globulin fraction of the protein. This fraction is precipitated by dialysis of the serum to remove the electrolytes, by dilution with distilled water, and by adjustment of the hydrogen-ion concentration to the point of maximum precipitation. The precipitated globulin is further purified by partial solution in dilute sodium chloride solution and reprecipitation in distilled water. The purified precipitate is dissolved in sodium chloride solution to give a final concentration of 0.8-per-cent salt, cresol to give a concentration of 0.3 per cent is added as preservative, and the reaction is adjusted to pH 7.4.

Pooling and Dialysis of Serum

Measure the volume of serum from each bleeding in a graduated precipitating jar and record. Pool the bleedings in a 75-liter glass-lined container. Distribute the serum in approximately 2500-ml. amounts in circular sheets of Cellophane, No. 600, 30 inches in diameter, and dialyze for four days against cold running tap water.

Precipitation of the Water-Insoluble Globulin

Pool and measure the volume of the dialyzed material. Rinse each bag with distilled water, add rinsings, and mix thoroughly. Into each of 5 tubes (150 by 15 mm.), pipette 3 ml. of the material and 6 ml. of distilled water. Add to the contents of the tubes, 0.05, 0.1, 0.2, 0.3, and 0.4 ml. respectively of 0.1 *N* hydrochloric acid and mix. In order to determine the amount that gives maximum precipitation of the specific protein, proceed as follows: Centrifuge the samples to throw down the resulting precipitates and decant the clear supernatants into clean tubes. To the latter add 3 drops of a 1:1000 dilution of the type-specific soluble carbohydrate. (In case the specific antigens are not available, the technic described under "Further Purification of Antitoxin by Isoelectric Precipitation" may be used.) After fifteen minutes note the tube in which there is the least evidence of precipitation. This indicates the presence of only a trace of specific protein and the optimum amount of acid for the separation of the active fraction. Calculate the necessary volume of N hydrochloric acid to be added to the main volume of material according to the following formula

$$\text{Vol. of } N \text{ HCl required} = \frac{\text{Vol. of material}}{3} \times \frac{\text{Vol. of } 0.1N \text{ HCl}}{10}$$

Stirring constantly, add the calculated amount of N hydrochloric acid slowly to the dialyzed material. Pour the adjusted material into approximately twice its volume of distilled water containing 0.2 per cent of cresol. (The water and cresol are measured into 75-liter glass-lined containers on the previous day and placed in the cold room to chill.) Repeat the test with the specific antigen to determine the completeness of precipitation. Readjust if necessary. Allow the precipitate to settle overnight in the cold room.

Purification of the Water-Insoluble Globulin

The method of purification is based on the fact that the specific fraction of the globulin is more soluble in dilute sodium chloride solution than is the inert protein.

Siphon the clear supernatant from the precipitated protein. Measure the volume of the precipitated residue. If necessary, add sufficient distilled water to bring the volume up to one-third of that of the original serum. This dilution facilitates sedimentation of the insoluble protein. Add sufficient sodium chloride to give a concentration of 0.4 per cent and preservative, at present phenylmercuric acetate to give a concentration of 0.001 per cent (10 ml. per liter of a 0.1-per-cent solution).

The concentration of sodium chloride added in this step is usually 0.4 per cent but it may be varied. Occasionally the insoluble residue is dissolved in a 1.5-per-cent sodium chloride solution and its potency determined. If the active material present in this fraction amounts to more than 10 per cent of that originally in the serum, the concentration of sodium chloride should be increased to more than 0.4 per cent.

Pour the material into 8-liter bottles and allow the undissolved protein to settle overnight at room temperature. Draw off the clear supernatant. Mix the residue with approximately one-half its volume of 0.4-per-cent sodium chloride solution, and centrifuge. Add the supernatant to the previous material and discard the residue. Reprecipitate the potent globulin fraction by pouring it with constant stirring into approximately five volumes of distilled water previously chilled. Allow the precipitate to settle overnight in the cold room. Draw off and discard the clear supernatant.

Collection and Solution of the Potent Globulin Fraction

Collect the protein precipitate by centrifuging the residue in a Sharples centrifuge. Start the centrifuge and regulate the flow of material to obtain a clear effluent. Observe the effluent at frequent intervals and when it becomes cloudy, stop the centrifuge, remove the bowl, and with a steel spatula scrape the precipitate from the inside into a graduated precipitation jar. Add a measured volume of 1.6-per-cent sodium chloride solution approximately equal to that of the precipitate. Repeat these operations until all of the precipitate has been collected. Stir until the precipitate is dissolved and note the volume. The final volume should be twice that of the 1.6-per-cent sodium chloride solution added, in order that the concentration of sodium chloride will be 0.8 per cent. Cresol to give a concentration of 0.3 per cent is added as a preservative. Emulsify the cresol in a separatory funnel with at least twice its volume of either the 1.6-per-cent sodium chloride solution or distilled water. Stir the protein solution constantly and add the cresol and then the remainder of either the sodium chloride solution or distilled water.

Adjust the reaction of the material to pH 7.4. Follow the procedure in "Neutralization of Antitoxin and Addition of Preservative," p. 721. In this instance the cresol is not emulsified with the sodium carbonate solution since the volume of the latter is usually too small.

Adjustment of Total Solids Content

Determine the total solids content of each lot of concentrated serum. Antipneumococcus serum is adjusted to between 8 and 10 per cent total solids; antimeningococcus serum to not more than 9 per cent. Generally, this procedure is carried out within the week following concentration. If potency tests indicate a sufficiently high titer, the sera may be diluted further.

Determination of "Unit Concentration" and Loss in Total Number of Units

See section under "Concentration of Antitoxic Plasmas," p. 722.

PURIFICATION AND CONCENTRATION OF ANTIPNEUMOCOCCUS RABBIT SERUM

In contrast to horse serum, very little of the specific activity of antipneumococcus rabbit serum is associated with the water-insoluble globulin fraction. While in horse serum only a small proportion of the antibody is precipitated by saturating the serum with sodium chloride, in rabbit serum a much greater part is associated with this fraction. After saturating the latter serum with sodium chloride, the addition of 7 per cent of sodium sulfate completes the precipitation of potent material.

Based on these facts, the following method of concentration has been developed. The water-insoluble globulin is separated by dialysis and isoelectric precipitation and discarded. The soluble fraction is neutralized, and 7 per cent of sodium sulfate and sodium chloride to saturation are added. The precipitated globulin is removed by filtration, pressed, and dialyzed. Sodium chloride to give a concentration of 0.8 per cent is added to the dialyzed material, 0.2 per cent of phenol and 0.005 per cent of 'Merthiolate' are added as preservative, and finally the reaction is adjusted to pH 7.4. This method has also been applied to the purification and concentration of antipertussis rabbit serum.

Pooling and Dialysis of Serum

Pool the serum and measure the total volume in a graduated glass precipitating jar. At present there is no definite limitation on the volume taken for concentration. Distribute the serum in circular sheets of Cellophane, No. 600, in amounts not exceeding 1000 ml. each and dialyze for three days against cold running tap water. In the case of small amounts of serum, the time of dialysis may be reduced to two days.

Precipitation of the Water-Insoluble Globulin

Pool the dialyzed material in a graduated precipitating jar. Rinse each bag with distilled water and add the rinsings. Measure the total volume and add an equal volume of distilled water. Mix thoroughly. Into each of 5 tubes (150 by 15 mm.) pipette 10 ml. of the material and add to the contents of the tubes 0.6, 0.7, 0.8, 0.9, and 1.0 ml. respectively of 0.1 *N* hydrochloric acid and mix. Proceed as in "Further Purification of the Antitoxin by Isoelectric Precipitation," p. 720. Calculate the volume of *N* hydrochloric acid necessary to adjust the main volume of material according to the following formula:

$$\text{Volume of } N \text{ HCL required} = \frac{\text{Volume of material}}{10} \times \frac{\text{Vol. } 0.1N \text{ HCl}}{10}$$

Stirring constantly, add the calculated amount of N hydrochloric acid. Allow the resulting precipitate to settle for several hours in the cold room, siphon off the clear supernatant, and centrifuge the residue. Discard the precipitate and combine the two supernatants.

Precipitation of the Active Globulin Fraction

Adjust the reaction of the soluble fraction to approximately pH 7.2 using the spot-plate method. As a start, add slowly a volume of N sodium hydroxide equal to one-half the volume of N hydrochloric acid used in the previous adjustment. Stir the solution constantly during this addition. Pipette several drops of the material into a hollow in a white porcelain spot plate and add one drop of phenol red indicator. Compare the shade produced with that of a buffer solution, pH 7.2, similarly treated. If the solution is too acid, continue adding N sodium hydroxide in small amounts and testing until the shade of the buffer solution is matched. Add 7 per cent (70 grams per liter) of anhydrous sodium sulfate. Solution of this salt may be facilitated by adding it slowly to the material while the latter is stirred vigorously. Saturate with sodium chloride by adding 335 grams per liter of this salt. Stir the solution until only a small residue of salt remains undissolved. Let the precipitated material stand for one hour and filter through hard filter paper, Whatman No. 50.

Drying of the Precipitate

Press the precipitate lightly. The pressing is not carried to the same extent as in the case of antitoxin and is usually completed in one day. No transfer is made of the precipitate from the filter paper to a muslin cloth.

Dialysis

Transfer the precipitate to Cellophane bags and dialyze three days against cold running tap water.

Neutralization. Addition of Preservative

Pool and measure the volume of the dialyzed material. Add sufficient sodium chloride to give a concentration of 0.8 per cent. Adjust the reaction to pH 7.4. Calculate the volume of 90-per-cent phenol necessary to give a concentration of 0.2 per cent. Dissolve the phenol in an approximately equal volume of ether, pipette it onto the surface of the material, and rotate the bottle until solution is completed. Then add sufficient 10-per-cent 'Merthiolate' to give a concentration of 0.005 per cent.

Adjustment of Total Solids Content

Determine the total solids content of each lot of concentrated serum and adjust to between 10 and 12 per cent.

Determination of "Unit Concentration and Loss in Total Number of Units"

See "Purification and Concentration of Antitoxic Plasmas," p. 722.

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CHAPTER 56

PREPARATION OF IMMUNE GLOBULIN SOLUTION (HUMAN)

Globulin solution for use in the modification or prevention of measles is prepared from human placentas (1, 2). The placentas, obtained from donors meeting specified physical requirements that correspond to the Federal regulations, are ground and the globulins extracted with 4-per-cent sodium chloride solution. They are precipitated from the clarified extract with ammonium sulfate, dialyzed, and the tissue globulins removed by acid precipitation. The remaining globulins are again precipitated with ammonium sulfate and dialyzed. The pH of the dialyzed material is adjusted to that of the blood, and sodium chloride to give an 0.8-per-cent concentration and preservative are added. Since there is no laboratory test available for determining the potency of globulin solution, it is necessary to rely on clinical reports for an evaluation of its efficacy (3).

Requirements for Donors.—The placentas are obtained only from hospitals of recognized standing. Each donor must be certified by a physician to be free from the toxemias of pregnancy and from diseases transmissible by blood transfusion, as far as can be determined from personal history and physical examination. In addition, acceptable serologic tests of the mother's blood made during the course of pregnancy must have shown no evidence of syphilis. No placenta should be used which shows gross evidence of disease or other abnormal condition.

Grinding and Extraction of Placentas.—Placentas received on the same day are ground together and the globulins extracted without delay. Extracts of those collected on different days are pooled only after the certificates for all the donors have been received.

Placentas are ground in a motor-driven meat grinder. Assemble the grinder and pour boiling water through it. With sterile forceps and scissors remove the umbilical cord and membrane, cut each placenta into several pieces, and grind. Collect the ground tissue and mix with 4-per-cent sodium chloride solution containing preservative, at present 'Merthiolate' in a concentration of 1:10,000, 500 ml. for each placenta. Cover the container and store in the cold room overnight. The following morning line a basket centrifuge head with a double layer of cheesecloth. Connect the draining chamber with an 8-liter bottle by rubber tubing. Pour in slowly the mixture of salt solution and tissue. Start the centrifuge and continue running until discharge ceases; then for each placenta pour in 250 ml. of 4-per-cent sodium chloride solution containing 'Merthiolate' in a concentration of 1:10,000. Continue running until drainage again ceases. Discard the extracted tissue. Clarify the extract by centrifugation and store in the cold room.

Precipitation of the Globulins.—After extracts of about 60 placentas have been collected, they are pooled and the volume measured. Add an equal volume of saturated ammonium sulfate solution and allow to stand for one hour. Filter through hard filter paper, wash the precipitate once with half-saturated ammonium sulfate solution, transfer it to cellophane bags, and dialyze for five days against cold running tap water. Pour the dialyzed material into a graduated precipitating jar. Rinse the bags with distilled water and add the rinsings. Note the total volume and add sufficient sodium chloride to give a concentration of 0.8 per cent.

Removal of Tissue Globulin.—Pipette 10 ml. of material into each of five tubes. Add to one tube 0.4 ml. of 0.1 N hydrochloric acid and to the others volumes increased progressively by 0.2 ml. Separate the precipitates by centrifugtion. To the clear supernatant from the first tube, add one drop of 0.1 N hydrochloric acid which should cause a precipitate to form. Test the supernatant in the second tube in the same manner and continue until a tube is reached in which the addition of acid produces no precipitate. If precipitates appear in all of the tubes, repeat the test with increased amounts of acid. Calculate the volume of N hydrochloric acid necessary to adjust the main volume of material as follows:

$$\text{ml. } N \text{ HCl required} = \frac{\text{Vol. of material}}{10} \times \frac{\text{Vol. of } 0.1N \text{ HCl used}}{10}$$

Add the required volume of acid, mix thoroughly, and immediately remove the resulting precipitate by centrifugation.

Reprecipitation of the Remaining Globulins.—Neutralize the supernatant with N sodium hydroxide using the spot-plate method and phenol red as indicator. Measure the total volume and add an equal amount of saturated ammonium sulfate solution. Allow to stand for one hour, filter through hard paper, press the precipitate, and dialyze for five days.

Neutralization and Addition of Preservative.—The dialyzed material is neutralized to pH 7.4 by the addition of sodium carbonate. Phenol and 'Merthiolate' are added as preservatives to give final concentrations of 0.2 per cent and 1:20,000 respectively.

Pool the dialyzed globulin solution and measure the volume. Add sufficient sodium chloride to give an 0.8-per-cent concentration. With vigorous stirring add for every 100 ml. of globulin solution, first, 4.16 ml. of 5-per-cent phenol, and then 0.5 ml. of 1-per-cent 'Merthiolate.' Finally adjust the reaction to pH 7.4, and store the material in the cold room.

Adjustment of Total Solids Content. Filtration.—Adjust the total solids content to between 5 and 7 per cent. The globulin solution is then filtered through infusorial earth and a final filter candle.

PREPARATION FOR DISTRIBUTION

After the sterility tests have been completed, the globulin solution is dispensed in 5-ml. volumes. If kept under proper conditions, the material is considered satisfactory for one year from the date of issue provided this is not later than two years after the extracts are pooled for concentration.

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CHAPTER 57

PREPARATION OF DIPHTHERIA TOXOID

Diphtheria toxoid is prepared for use in the active immunization of persons against diphtheria by subjecting potent diphtheria toxin to the action of formalin and heat until it is detoxified. Toxoid is prepared routinely from infusion-free toxin; occasionally, for comparative purposes, from veal-infusion toxin (1).

Preparation of Toxin

Toxins without preservative are used for the preparation of toxoid. They are filtered into graduated bottles. The minimum lethal dose for guinea pigs and the flocculation value are determined for each lot of toxin. See "Production and Standardization of Diphtheria Toxin," p. 623.

PREPARATION OF TOXOID

Immediately following filtration, with aseptic precautions, pipette formalin into each bottle of toxin, mix thoroughly by rotating the bottle, and incubate at 38°–39°C. Except during incubation, keep the toxoid in the cold room.

The percentage of formalin and the period of incubation necessary for detoxification depend upon the type of medium used in the preparation of a given toxin. At present, the following give satisfactory results: maltose sodium-acetate infusion-free medium (*F20*)—0.24 per cent of 40-per-cent formalin and incubation for twelve days for strain No. 5, seven days for strain No. 3203; maltose sodium-lactate infusion-free—0.24 per cent with twenty-two days' incubation; veal broth (*F12*) toxin—0.4-per-cent formalin and incubation for six weeks or more.

Detoxification Test.—At the end of the usual incubation period, remove samples from each bottle for tests of detoxification, and make the required sterility tests. If a lot consists of 4 bottles or more, inject 2 guinea pigs with material from each bottle, otherwise 3. Use animals weighing from 230 to 280 grams; clip the hair at the site of injection. Allow the toxoid to reach room temperature, then inject subcutaneously 5 ml., 3 ml. on one side of the abdominal region and 2 ml. on the other. To prevent oozing, apply a drop of collodion to each site.

Observe the animals for thirty-five days for evidence of diphtheria poisoning and weigh them on the 5th, 10th, 20th, and last day. Both local reactions and paralysis should be absent or practically so. If the results of the tests indicate insufficient detoxification (early local reactions or late paralysis), return the bottles to the incubator for a few days or weeks, depending upon the type of material and the severity of the reactions, then repeat the tests.

Addition of Preservative.—When the tests indicate adequate detoxification, and the material is not to be precipitated, add 10-per-cent 'Merthiolate' to give a final concentration of 0.01 per cent. Mix thoroughly by rotating each bottle. Remove samples for the final flocculation and animal standardization tests, and make the required tests for sterility.

STANDARDIZATION OF TOXOID

Diphtheria toxoid is standardized by determining its immunizing value for guinea pigs and its Lf value.

Flocculation Test.—Test the toxoid by the flocculation method (see p. 628). The flocculation value is usually equal to or slightly less than that of the original toxin but the reaction time is longer.

Animal Test.—Inject subcutaneously 0.5 ml. of toxoid from one bottle of each lot into each of 5 guinea pigs weighing from 270 to 320 grams. Thirty days later inject subcutaneously each animal with 5 M.L.D. of potent diphtheria toxin. At the same time, as a control of the potency of the toxin, inject two normal guinea pigs weighing from 230 to 280 grams with one M.L.D. At least 80 per cent of the immunized animals should survive for ten days and the control animals should die within ninety-six hours.

PREPARATION FOR DISTRIBUTION

Toxoid is dispensed in 2- and 5-ml. volumes. If kept under proper conditions, the material is considered satisfactory for use for two years from the date of the last satisfactory antigenicity test in animals.

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CHAPTER 58

PREPARATION OF TETANUS TOXOID

Tetanus toxoid is prepared for the active immunization of persons and horses against tetanus by subjecting potent tetanus toxin to the action of formalin and heat until it is detoxified. The procedures are similar to those used for the preparation of diphtheria toxoid.

Preparation of Toxin

Toxins without preservative are used for the preparation of toxoid. They are filtered into graduated bottles. The minimum lethal dose for guinea pigs and the L+ dose are determined for each lot of toxin. See "Production and Standardization of Tetanus Toxin," p. 631.

PREPARATION OF TOXOID

Immediately following filtration, with aseptic precautions, pipette formalin into each bottle of toxin, mix thoroughly by rotating the bottle, and incubate at 36°–37°C. At present, sufficient 40-per-cent formalin is added to give a final concentration of 0.3 per cent; the incubation period is twenty-two days.

The percentage of formalin and the period of incubation necessary for detoxification are the same for toxins produced in autolysate media with either beef-heart or veal infusion.

Detoxification Test.—The test is performed as described for diphtheria toxoid, see p. 734. Use guinea pigs weighing from 330 to 380 grams, and observe the animals for twenty-five days and weigh them on the 5th, 10th, 20th, and last day. Be especially alert to detect any evidence, however slight, of spastic paralysis. If any symptoms of tetanus develop, return the bottles to the incubator for a few days, depending upon the severity of the symptoms, then repeat the tests.

Addition of Preservative.—See "Diphtheria Toxoid," p. 735.

STANDARDIZATION OF TOXOID

Tetanus toxoid is standardized by determining its immunizing value for guinea pigs.

Procedure.—Inject subcutaneously 1 ml. of toxoid from one bottle of each lot into each of five guinea pigs weighing from 330 to 380 grams. Thirty days later inject each animal subcutaneously with 10 M.L.D. of potent tetanus toxin. At the same time, as a control of the potency of the toxin, inject two normal guinea pigs weighing from 330 to 380 grams with 1 M.L.D. At least 80 per cent of the immunized animals should survive for ten days and show no symptoms of tetanus. The control animals should die within ninety-six hours.

PREPARATION FOR DISTRIBUTION

Toxoid is dispensed in 2- and 10-ml. volumes. If kept under proper conditions, the material is considered satisfactory for use for two years from the date of the last satisfactory antigenicity test.

CHAPTER 59

PREPARATION OF DIPHTHERIA TOXOID—PRECIPITATED

Precipitated diphtheria toxoid is prepared by adsorbing the antigenic fraction of potent toxoid on a precipitate formed under suitable conditions by the addition to the toxoid of an optimum concentration of aluminum potassium sulfate ($AlK(SO_4)_2 \cdot 12H_2O$) (1, 2). It is used for the active immunization of children (preferably not more than fifteen years of age) against diphtheria.

PREPARATION OF PRECIPITATED TOXOID

Toxoid.—Toxoid without preservative is used. For preparation, see p. 734.

Toxoid prepared by the action of formalin in 0.9-per-cent concentration and incubation for a short period at 36°C. has also been used (3). The toxoid is precipitated immediately upon removal from the incubator. As the final product must be discarded if too toxic, it is necessary to determine carefully the incubation period for each type of toxin. Tests for detoxification, see p. 734, are made on a sample of the precipitated material taken before the addition of preservative. Toxins prepared with *Corynebacterium diphtheriae* strain No. 5 in maltose sodium-lactate medium required ninety hours' incubation for detoxification; toxins of strain No. 3203 in maltose sodium-acetate medium, sixty-six hours.

Precipitating Solution.—Aluminum potassium sulfate (C.P.) is used in a 10-per-cent solution. The solution is sterilized by filtration through a Mandler filter candle into resistant glass bottles. Usually a supply of twenty-four liters is prepared at one time.

Procedure

The closed system of siphoning is used throughout. See "General Instructions," p. 621. At present sufficient aluminum potassium sulfate solution is added to the toxoid to give a concentration of approximately 1.5 per cent (175 ml. of solution per liter of toxoid). Siphon the calculated amount of alum solution into graduated bottles (one for each lot of toxoid to be precipitated), pipetting any amount necessary to make the exact volume. When the toxoid has reached room temperature, siphon it into graduated resistant glass bottles (usually five-liter amounts in eight-liter bottles). Insert a siphon into the measured volume of alum solution, connect it with the bottle containing the toxoid, transfer the toxoid bottle to the mixing apparatus (Fig. 66), and while the bottle is being rotated,

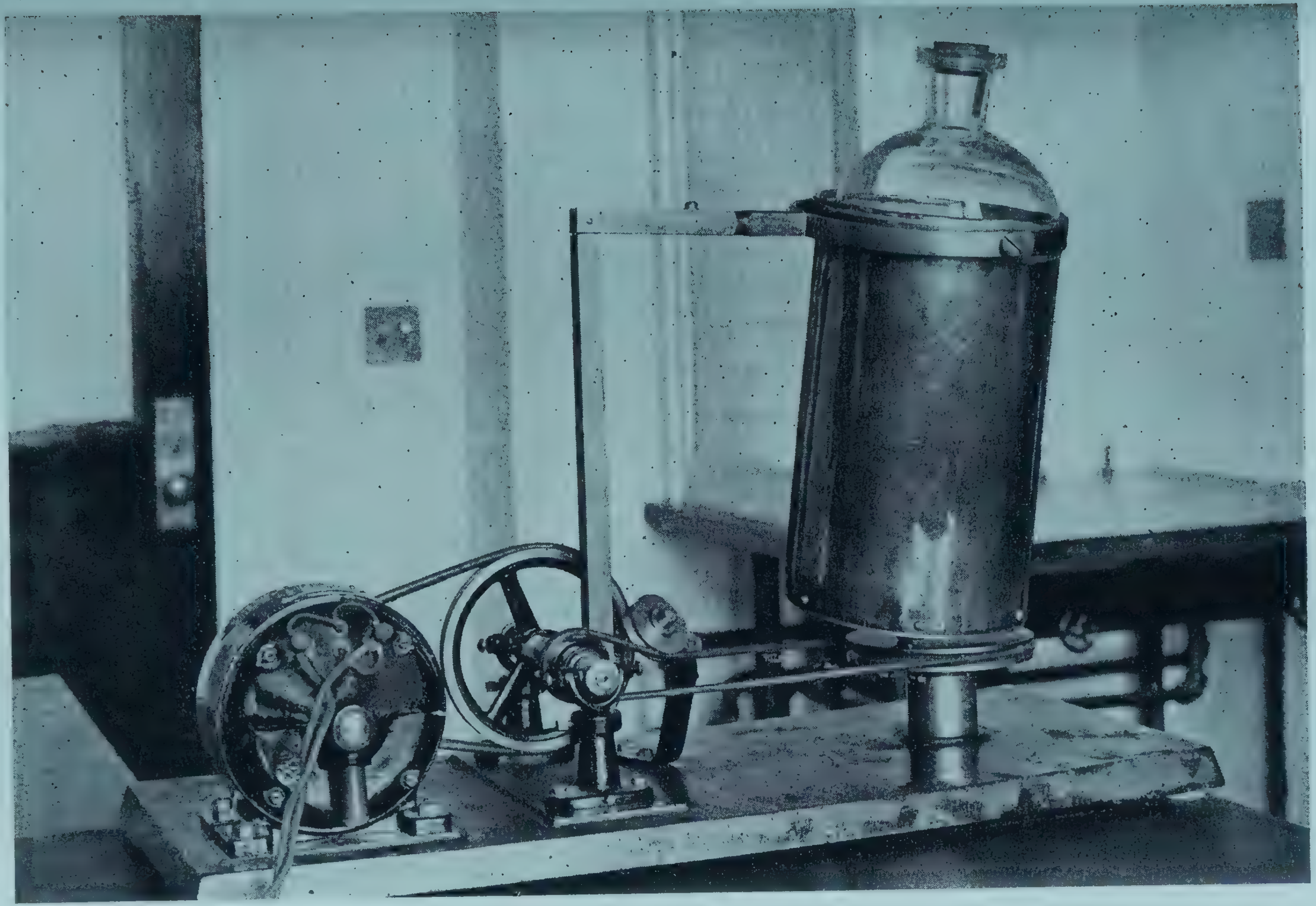

FIG. 66. MIXING APPARATUS: DESIGNED TO MIX WITHOUT UNNECESSARY AGITATION

A copper container $7\frac{3}{4}$ inches by 12 inches (inside dimensions) held at the top by a hinged universal, and driven from below by a crank. The crank pin is a ball fitting in a socket attached to the center of the bottom of the container, and adjustable on the pulley to three different throws. The machine is driven at a speed of 140 r.p.m. by a $\frac{1}{6}$-horse-power motor. The belt is held taut by an idler and spring. The apparatus is mounted on a cast-iron base.

add the alum solution to the toxoid slowly. Continue mixing in the machine for at least one minute. Allow the precipitate to settle in the cold room for three and one-half hours. Connect the special siphon system for aseptic washing of precipitates (for preparation, see p. 621) with the end of the siphon about 4 cm. above the level of the precipitate. Draw off the supernatant, tipping the bottle carefully to remove as much as possible. Place a pinchcock on the outlet tube, and through the inlet tube siphon sterile salt solution (rotating the bottle at intervals to break up the precipitate) to as high a level as possible, allowing space for adequate mixing of the suspended precipitate. After rotating the bottle vigorously, place the bottles still connected in the cold room.

Early the next morning and again in the late afternoon draw off the supernatant and wash the precipitate. Return the bottles to the cold room each time. The third morning repeat, but add only sufficient salt solution to make the volume equal to that of the fluid toxoid. Add sufficient 10-per-cent solution of 'Merthiolate' to give a final concentration of 0.01 per cent and rotate the bottle vigorously. Take samples from each lot for animal and flocculation tests, and make the required sterility tests. Each amount of toxoid to which aluminum potassium sulfate is added represents a separate lot and is tested accordingly. To ensure that each sample is representative of the suspension, use a separate pipette for each, rotate the bottle immediately before inserting, and empty the entire contents of a pipette into one bottle.

Divide the contents of a bottle into 8-liter resistant glass bottles so that each contains not more than 4 liters of infusion-free toxoid or 3 liters of veal-broth toxoid since a large amount of foam is produced by air agitation when the toxoid is dispensed. Place connecting siphons in the bottle containing the precipitated toxoid and in the empty bottle. While the toxoid is rotated vigorously in the mixing machine, transfer the desired amount. Make a sterility test on each bottle. Depending upon how and when the precipitated material is to be used, this procedure may be carried out either at the end of the precipitation or before it is dispensed for distribution.

An adjustment of pH is required to obtain optimum conditions for the formation of an adequate precipitate when aluminum potassium sulfate is added to toxoids prepared in maltose sodium lactate infusion-free medium (see method 2, p. 744).

Precipitated toxoid has also been prepared by adsorption on a precipitate of $Ca(PO_4)_2$ (3). In general, the procedures are similar to those followed when aluminum potassium sulfate is used.

While the bottle of toxoid is rotated, add a sufficient amount of a 10-per-cent solution of $CaCl_2$ to give a final concentration of 1 per cent and twice this amount of a 10-per-cent solution of sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$). Allow the precipitate to settle and wash twice with sterile distilled water and three times with salt solution. Wash until the supernatant contains less than 0.05-per-cent $CaCl_2$.

Restore the precipitate to the original volume of toxoid and proceed as with the alum-precipitated material.

STANDARDIZATION OF PRECIPITATED TOXOID

FLOCCULATION TEST

The flocculation, Lf, value is usually slightly lower than that of the original toxoid and the flocculation time considerably longer. Add to 100 ml. of a suspension of alum-precipitated toxoid 2 grams of sodium citrate and heat in a water bath at 40°–45°C. until the precipitate is dissolved and the liquid has become clear, usually for from twenty to thirty minutes. Allow the solution to come to room temperature and proceed to the flocculation test, see p. 628.

The calcium precipitated toxoid is dissolved at room temperature by the addition of 10 ml. of a 20-per-cent solution of diammonium citrate per 100 ml.

TEST IN GUINEA PIGS

For tests of antigenicity, inject 0.5 ml. of uniform suspension into each of 5 guinea pigs, preferably males, weighing between 470 and 520 grams. Observe local and general reactions, and weigh the animals on the 5th, 10th, 20th, and 30th day. On the 30th day, take from 8 to 10 ml. of blood from the heart of each guinea pig, omitting any with evidence of infection. Pool equal volumes of the sera of animals immunized with a given lot of toxoid. Test the pooled sera by the subcutaneous method for diphtheria antitoxic content (usually 2, 3, and 4 or more units per ml.). See "Production and Standardization of Diphtheria Antitoxin," p. 654.

The titer of the serum is estimated by comparing the time of death of the animals injected with the different doses of test serum with that of the animals injected with the standard control. The pooled sera should contain at least two antitoxic units per milliliter if the material is to be used for pooled diphtheria-tetanus toxoid. In case the sera are found to contain less than two units of antitoxin, the toxoid is retested similarly in guinea pigs using a 1-ml. dose. If the pooled sera from the guinea pigs contain at least two antitoxic units per milliliter, the material is considered satisfactory for use as percipitated diphtheria toxoid.

PREPARATION FOR DISTRIBUTION

Precipitated diphtheria toxoid is dispensed in 2.5- and 10-ml. volumes. Special procedures are followed in dispensing the toxoid to ensure uniform distribution of the precipitate. For details, see methods for the preparation of products for distribution, p. 824. At present precipitated diphtheria toxoid, if kept under proper conditions, is considered satisfactory for use for two years from the date of the last satisfactory test. The date that guinea pigs producing serum of 2 or more antitoxic units per milliliter were injected is considered the date of a satisfactory test.

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CHAPTER 60

PREPARATION OF DIPHTHERIA-TETANUS TOXOID—PRECIPITATED

Diphtheria-tetanus toxoid, precipitated, is prepared by mixing equal parts of each of the precipitated toxoids. It is used for the active immunization of children (preferably not more than fifteen years of age) against diphtheria and tetanus.

PREPARATION OF PRECIPITATED DIPHTHERIA TOXOID

See section under "Preparation of Diphtheria Toxoid—Precipitated," p. 738.

PREPARATION OF PRECIPITATED TETANUS TOXOID

Precipitated tetanus toxoid is prepared by adsorbing the antigenic fraction of potent tetanus toxoid on a precipitate formed under suitable conditions by the addition to the toxoid of an optimum concentration of aluminum potassium sulfate ($AlK(SO_4)_2 \cdot 12H_2O$).

Toxoid.—Toxoid without preservative is used. For preparation, see p. 736.

Precipitating Solution.—Aluminum potassium sulfate (C.P.) is used in a 10-per-cent solution. For preparation, see p. 738.

Procedure

The procedures for precipitation are the same as those for diphtheria toxoid, except that an adjustment of pH is required to obtain optimum conditions for the formation of an adequate precipitate with aluminum potassium sulfate. Two methods have proved satisfactory (1). The first is preferred since the precipitate produced by this method settles more rapidly.

1. Siphon the toxoid and sufficient 10-per-cent aluminum potassium sulfate solution to give a final concentration of 1.5 per cent, as described on p. 738. While the mixture is still being rotated, add sufficient sterile 15-per-cent sodium hydroxide to give a pH between 5.5 and 6.5 (from 18 ml. to 22 ml. per liter of toxoid). The NaOH solution is added through a sterile cylindrical funnel the upper end of which is plugged with cotton and the stem connected to the bottle containing the toxoid by rubber and glass tubing. Rotate the bottle of toxoid while the NaOH is added. After adjustment of the pH, proceed as for precipitated diphtheria toxoid.

2. Using the same procedures as in method 1, siphon the toxoid into graduated bottles. Take an aliquot sample and determine electrometrically the amount of 15-per-cent NaOH required to bring the pH to approximately 8.5. Calculate the amount of NaOH required for the total volume and add as in method 1. Siphon into the adjusted toxoid sufficient 10-per-cent aluminum potassium sulfate solution to give a final concentration of 1.5 per cent and continue as for precipitated toxoid.

Standardization of Precipitated Tetanus Toxoid

Test in Guinea Pigs.—For tests of antigenicity, inject five guinea pigs, preferably males, weighing between 470 and 520 grams, with 0.5 ml. of uniform suspension and bleed after six weeks. Follow the procedures given for tests of precipitated diphtheria toxoid, see p. 741. Weigh the animals as required and in addition on the 42d day. Test the pooled sera by the subcutaneous method for tetanus antitoxic content (usually 2, 3, and 4, or more units per ml.). See "Production and Standardization of Tetanus Antitoxin," p. 662.

The titer of the serum is estimated by comparing the time of death of the animals injected with the different doses of test serum with that of the animals injected with the standard control. The pooled sera should contain more than two antitoxic units per milliliter.

PREPARATION OF PRECIPITATED DIPHTHERIA-TETANUS TOXOID

At present, equal volumes of precipitated diphtheria and tetanus toxoids are pooled. The closed system of siphoning is used. Place connecting siphons in the bottle from which one of the precipitated toxoids is to be delivered and in an empty bottle or in the one containing a measured volume of the other precipitated toxoid. While the first bottle is rotated vigorously in the mixing machine, transfer the required amount of material to the other bottle. Mix thoroughly the pooled toxoids, take samples from each lot for animal tests, and make the required sterility tests. To ensure that each sample is representative of the suspension, use a separate pipette for each, rotate the bottle immediately before inserting, and empty the entire contents of a pipette into one bottle.

STANDARDIZATION OF PRECIPITATED DIPHTHERIA-TETANUS TOXOID

Test in Guinea Pigs.—For tests of antigenicity, inject ten guinea pigs, preferably males, weighing between 470 and 520 grams with 1 ml. of uniform suspension. Bleed five of the animals 30 days after injection and test the pooled sera for diphtheria antitoxic content. Bleed the remaining five 6 weeks after injection and test the pooled sera for tetanus antitoxic content. Follow the procedures described for antigenicity tests of the separate precipitated toxoids. The pooled sera from the first group of animals should contain at least 2 diphtheria antitoxic units per ml.; that from the second group, at least 2 tetanus antitoxic units per ml.

PREPARATION FOR DISTRIBUTION

When the standardization tests are completed, siphon the precipitated toxoid into 8-liter resistant glass bottles as described for precipitated diphtheria toxoid, see p. 740.

Precipitated diphtheria-tetanus toxoid is dispensed in 2.5- and 10-ml. volumes. Special procedures are followed to ensure uniform distribution of the precipitate. For details, see methods for the preparation of products for distribution, p. 825. At present, precipitated diphtheria-tetanus toxoid, if kept under proper conditions, is considered satisfactory for use for two years from the date of the last satisfactory test. The date that guinea pigs producing serum of 2 or more antitoxic units per milliliter were injected is considered the date of a satisfactory test. If the dates of the satisfactory tests of the two toxoids in the mixture are different, the expiration date is based on the earlier test.

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CHAPTER 61

PREPARATION OF DILUTED STREPTOCOCCUS TOXIN FOR ACTIVE IMMUNIZATION

Streptococcus toxin is prepared for the active immunization of persons found by the intracutaneous test to be susceptible to the toxin. While its general use is not recommended, the toxin has proved of value as an immunizing agent in certain instances, such as in outbreaks of scarlet fever in institutions or in the case of nurses in training.

Limited trial of different preparations of formalinized streptococcus toxin containing 1000 skin test doses (S.T.D.) per milliliter failed to demonstrate their efficacy as immunizing agents on the basis of the results of the intracutaneous test of susceptibility. The antigenic value of tannic-acid precipitated toxin prepared according to the methods of Veldee (1) for intracutaneous injection in small doses is under investigation.

PREPARATION OF TOXIN DILUTIONS

Toxin: At present a stable toxin prepared with the standard strain, No. 165, aged for not more than one year, accurately standardized, and containing preferably over 40,000 S.T.D. per milliliter is used.

Diluent: Salt solution is used as the diluent. The amounts required are dispensed and sterilized in graduated bottles.

Procedure.—Five dilutions are prepared, the first containing 1000 S.T.D. per milliliter; the second, 4000; the third, 8000; the fourth 25,000; and the fifth 40,000.

Add to the salt solution sufficient 90-per-cent phenol solution (prepared by weight, sp. gr. 1.0677, 4.15 ml. per 1000 ml.) to give a final concentration of 0.4 per cent. Replace the loss by evaporation during sterilization with sterile distilled water. Add the calculated volume of toxin with a sterile pipette or siphon, depending upon the volume. For preparation of the siphon, see "General Instructions," p. 620. After each addition mix thoroughly by rotating the bottle. Remove a sample from each bottle for potency tests on animals and make the required sterility tests. If the results of the tests are not satisfactory, the material is discarded.

STANDARDIZATION OF TOXIN DILUTIONS

The sample from each dilution is tested for potency on animals in comparison with the standard toxin. See "Production and Standardization of Streptococcus Toxin," p. 641.

PREPARATION FOR DISTRIBUTION

When the sterility and potency tests are completed, the diluted toxin is dispensed in 10-ml. volumes. If kept under proper conditions, it is considered satisfactory for use for one year from the date of the potency test.

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CHAPTER 62

PREPARATION OF TYPHOID AND TYPHOID-PARATYPHOID VACCINES

Typhoid and combined typhoid and paratyphoid-A and -B vaccines are prepared for prophylactic use from selected strains of high antigenic value. Cultures grown on beef-infusion agar are suspended in salt solution, the suspension is heated, and cresol is added to give a 0.3-per-cent concentration. The final preparation of typhoid vaccine contains 1000 million microorganisms per milliliter, 500 million of each of two strains; that of typhoid-paratyphoid vaccine contains 1500 million, 500 million of each of two typhoid strains, and 250 million each of paratyphoid-A and paratyphoid-B strains. The immunizing activity of the vaccines is gauged by protection tests in mice.

The preparation of typhoid-paratyphoid vaccine was discontinued in October, 1937, because of the apparently low incidence in New York State of *Salmonella paratyphi*-A and -B infections. Preparation was resumed in 1941 to meet special situations during the war period.

Strains

Bacterium typhosum. Collection No. 62. "Rawlings" strain, isolated in 1903, and maintained in this laboratory since 1918. The colonies on beef-infusion agar plates after twenty-four hours' incubation are relatively small, usually with a slight dome, smooth or slightly rough surface, slightly undulate edges, and of a moist and homogeneous consistency. Cultures in broth show a uniform turbidity with a slight amount of sediment and a very fine pellicle. The bacilli are actively motile and are, in general, short rods. This strain has been retained because of its established antigenic activity and low toxicity.

Bact. typhosum. Collection No. 40494. Isolated from a chronic human carrier in Panama. Received in 1940 from the Army Medical School, Washington, D. C. (42–A–58) (1).

Salmonella paratyphi-A. Collection No. 42406. Received in 1942 from the Army Medical School, Washington, D. C. (41–N–22). Obtained originally from Dr. A. Felix, London (2).

S. paratyphi-B. Collection No. 41333. Received in 1941 from the Army Medical School, Washington, D. C. (41–H–6) (2). Obtained originally from the American Expeditionary Force in Siberia in 1920.

Maintenance of Strains.—The strains are maintained in the dry state. See "Maintenance of the Collection of Type Cultures," p. 90. Prepare dried cultures as required, usually every two or three years. The identity and purity of the cultures are sufficiently controlled by the preliminary tests made in connection with each lot of vaccine. Strain No. 62 is also passed through mice from time to time, usually just before the preparation of a lot of vaccine.

Subculture a dried preparation of the latest mouse-passage culture. Add 0.5 ml. of beef-infusion broth to the dried culture and inoculate from the suspension a beef-infusion agar slant (*F34A*). Incubate the culture overnight at 34°–36°C. From this culture inoculate a tube of beef-infusion broth and one of double-sugar beef-extract agar (*F50B*), slant and butt, and make a slide preparation stained by Gram's method. Incubate the cultures for eighteen hours. If they appear typical and there is no evidence of contamination, store the double-sugar agar culture in the cold room and inject two mice weighing from 18 to 22 grams, one with 0.5 and the other with 0.25 ml. of the broth culture. At autopsy, from the heart's blood inoculate a beef-infusion agar slant and an Endo agar plate (*F52A*). Examine the cultures recovered from the heart's blood of each mouse and, as a control, the previous mouse-passage culture for morphologic and cultural characters and for biochemical and serologic reactions. If there is any evidence of dissociation in the most recent mouse-passage cultures, discard them, and use the preceding one in the current lot of vaccine. If the cultures recently recovered from mice are satisfactory, select one, make dried preparations of it for storage, and proceed with the preparation of vaccine.

PREPARATION OF VACCINE

All procedures for the preparation of the vaccine are carried on in a room designed for rigidly aseptic technic. Special precautions are observed when live cultures of typhoid and paratyphoid microorganisms are handled (see "General Bacteriologic Technic," p. 4). All vaccine cultures are incubated at 34°–36°C.

The beef-infusion agar on which the cultures are grown is allowed to harden in 1-liter Roux culture bottles laid on their sides. They are incubated in an inverted position for forty-eight hours before inoculation to insure sterility and to facilitate the collection and removal of the "water of condensation." Usually from 30 to 40 bottles are inoculated with each typhoid strain, from 5 to 10 with each paratyphoid strain.

Supplies. Media: Two-per-cent beef-infusion agar (*F34A*), pH about 7.2, in 140-ml. volumes in 1-liter Pyrex Roux bottles with neck and cotton plug protected by a paper cap.

Swabs: Swabs of nonabsorbent cotton on 14-inch stainless-steel wire rods in copper pipette containers.

Pipettes: Graduated and bulb pipettes of different capacities in separate containers.

Scrapers: Stainless-steel rods, $\frac{3}{32}$ inch in diameter, 14 inches long and terminating in a triangular loop from $\frac{1}{2}$ to $\frac{5}{8}$ inch inside diameter, with the base ground to a dull edge, for scraping the growth from the surface of the agar cultures. After use, boil the scrapers in 5-per-cent sodium carbonate solution for five minutes, rinse, and dry.

Bottles: One-liter Blake bottles, graduated at 25-ml. intervals for pooling the concentrated suspension.

Salt solution: Salt solution dispensed in 1-liter volumes in 4-liter Pyrex bottles for washing off the growth; also, in 4-liter bottles in the volumes required for diluting the vaccines.

Distilled water: Dispensed in 2- or 3-liter volumes in 4-liter Pyrex bottles.

Siphons: Siphons made of glass and rubber tubing equipped with bell-covered delivery tips for dispensing (1) salt solution used in washing off the growth and (2) distilled water to replace the volume of liquid lost during sterilization in the bottles containing salt solution for diluting the vaccines.

The swabs, pipettes, scrapers, siphons, and bottles are sterilized the day before they are required; the salt solution, within two or three days.

Preliminary Transfers. Identification and Purity Tests.—Begin work with the two typhoid strains on succeeding days; with the paratyphoid strains on the third day. Several days before a lot of vaccine is to be begun, subculture a dried preparation of each strain. For special tests of strain No. 62, see p. 749. From the suspension of the dried culture, inoculate a beef-infusion agar slant (*F34A*). Incubate the culture overnight. Examine the agar slant, inoculate from it a tube of double-sugar beef-extract agar (*F50B*), slant and butt, and make a slide preparation stained by Gram's method. Store the agar slant in the cold room. If there is no evidence of contamination, proceed with the preliminary steps in the preparation of vaccine.

1st day. From the recent subculture of a dried preparation, transfer a loopful of the growth to a tube of beef-infusion broth and mix thoroughly. Streak one loopful of this suspension on one plate of Endo agar (*F52A*) and two of beef-infusion agar. Incubate for from eighteen to twenty-two hours.

2d day. Examine growth carefully and from each of three typical, distinct colonies on the beef-infusion agar inoculate two tubes of beef-infusion broth and one of double sugar, slant and butt. Incubate for from sixteen to eighteen hours.

3d day. If growth is typical, take a broth culture from two of the sets for motility tests and macroscopic agglutination tests with the homologous serum.

Test the cultures in dilutions of serum chosen according to the known titer of the serum, usually the range is from 1:100 to 1:4000. Prepare the dilutions with salt solution, and use salt solution and normal horse serum (1:100) as controls. Add 0.3 ml. of the broth culture to 0.3 ml. of salt solution and to similar amounts of the serum dilutions. Shake and incubate at 48°-52°C. for two hours except in the case of tests of strain No. 40494. Incubate these tests at least four hours.

From the duplicate broth culture of the set that agglutinates in high dilutions of the serum, is actively motile, and is most typical in appearance and reactions on double-sugar medium, inoculate two more than half as many agar slants (150- by 19-mm. tubes) as there are flasks to be inoculated. Incubate for from sixteen to eighteen hours. From the broth culture used for the seed, make a slide preparation stained by Gram's method.

4th day. Examine the cultures. Pipette 1 ml. of beef-infusion broth into each tube. Discard any cultures that agglutinate spontaneously or appear contaminated and proceed at once with the inoculation of the flasks.

Inoculation and Incubation of Cultures.—Remove the bottles from the incubator. Examine without turning and discard any that appear unsatisfactory. If necessary, pipette off the excess "water of condensation." Inoculate two bottles in succession from the suspension in one tube. Pass a swab soaked in the suspension quickly over the entire agar surface. Recharge the swab and inoculate the second bottle. Take a fresh swab for every two bottles. Place the contaminated swabs and pipettes in a jar containing a 2-per-cent cresol-compound solution. Incubate the bottles for from eighteen to twenty-two hours.

Harvesting of Growth.—5th day. Examine the bottles carefully and discard any that show definite contamination. Should unusual colonies be observed, make slide preparations from them and stain by Gram's method.

Set up a siphon, equipped with a delivery tip protected by a glass bell, in a 4-liter bottle containing salt solution and dispense about 15 or 20 ml. of the solution into each bottle. With a scraper, one for each bottle, loosen the growth, taking care not to break the surface of the agar. Make a slide preparation and stain. Discard bottles in which contamination is found or suspected and in which spontaneous agglutination occurs. Transfer the suspensions to a graduated 1000-ml. bottle. If pipettes are used, take a fresh one for every 6 or 8 suspensions. Replace the cotton plug in the collecting bottle with a sterile rubber stopper and shake the bottle vigorously to insure uniform suspension. Remove immediately about 2 ml. for the bacterial count, then cap the bottle with 4 layers of fishskin wet with 1-per-cent cresol.

Heating of Suspension.—Use a water bath equipped with a thermoregulator, a perforated tray raised from the bottom, and an electric stirrer to maintain the water at a constant and uniform temperature throughout the bath. Heat the water to 53°C. Submerge the bottle of vaccine and also a control bottle containing water with a thermometer in it. After the thermometer in the control bottle registers 53°C., hold at this temperature for one hour, then remove the bottle of vaccine.

Bacterial Count.—The standardization of typhoid and paratyphoid vaccines by turbidity measurements made with a photoelectric instrument is being studied. This method, or one based on the nitrogen content of the vaccines, may later be adopted. In the meantime, the direct count is retained until sufficient data are obtained on suspensions of each of the four vaccine strains in use.

Make the bacterial count by means of a Helber counting chamber while the suspension is being heated. If the count is delayed, place the sample in the cold room. Dilute the vaccine usually 1:50 or higher depending upon the density of the suspension. Prepare three dilutions of the same strength and make at least one count from each.

Pipette about 1 ml. of the diluted suspension into a clean, dry watch glass. In a white blood cell diluting pipette draw up the bacterial suspension exactly to the 0.5 mark, then freshly filtered staining solution (1:200 dilution of carbolfuchsin stain in a 5-per-cent phenol solution) to the 11 mark. If a Trenner automatic blood diluting pipette is used, draw the bacterial suspension to the bulb and the stain to the graduation above the bulb. Slip a wide rubber band over the ends of the pipette and mix by shaking for several minutes. Do not permit the suspension to stand in the pipette. Discard a few drops, then transfer one to a Helber counting chamber. The drop should just fill the center area so that there are no bubbles and no excess fluid to run over into the safety trench.

A binocular microscope equipped with 10x oculars and an objective 40 n.a. 0.65 is used. In order to include all of the bacilli in a given field, focus on different levels. Count at least 20 squares. Beginning with the first completely ruled square at the upper felt-hand corner, count diagonally across the counting chamber; then count a few additional squares from sections of the chamber not already included. Determine the average number in a square. If the counts of the 3 dilutions agree within about 10 per cent, take the average as the count of the vaccine; otherwise, make additional dilutions, count, and take the average of all. Multiply the number of bacilli in one square by 400 million to obtain the number in one milliliter of dilution and then by the original dilution factor used, in order to obtain the number of bacilli per milliliter of suspension.

The figure 400 million is derived as follows: cubic contents of each small square in counting chamber:

$$1/20 \text{ mm.} \times 1/20 \text{ mm.} \times 1/50 \text{ mm.} = 1/20{,}000 \text{ cmm.} = \text{approximately } 1/20{,}000{,}000 \text{ ml.}$$

$$1/20{,}000{,}000 \times (1/20, \text{dilution in pipette}) = \text{approximately } 1/400{,}000{,}000 \text{ ml.}$$

Purity Test on Heated Suspension.—Just before adding the preservative, remove a small amount of the heated suspension with a pipette and streak two blood-agar plates. Some growth will develop but there should be no contaminating microorganisms.

Addition of Preservative to Concentrated Suspension.—A 1.5-per-cent solution of cresol freshly prepared in salt solution is added to the suspension to give a final 0.3-per-cent concentration. Calculate the volume to be added, pipette it into the bottle of vaccine, and mix thoroughly. Determine the volume of preservative to be added as follows:

$$\text{Vol. of preservative to be added} = \frac{\text{(required preservative concentration)(vol. of vaccine to be treated)}}{\text{concentration of preservative sol. used} - \text{required concentration}}$$

In practice, multiply the volume of vaccine by 0.25, a constant as long as a 1.5-per-cent solution of cresol is added to give a 0.3-per-cent concentration. The addition of preservative necessitates correction of the bacterial count. Since the preservative is added in a quantity equal to one-quarter the volume of the vaccine, multiply the number of bacilli per milliliter by four-fifths to obtain the revised count.

Sterility Tests of Concentrated Suspensions.—Store the cresolized suspension in the cold room after removing a 5-ml. sample. Hold the sample at room temperature for about eighteen hours so that any viable bacilli may be killed more rapidly by the action of the preservative. Inoculate one tube each of aerobic and anaerobic "sterility-test" broth (*F3B*) with 0.5 ml. of the concentrated vaccine sample and prepare a tube of deep glucose beef-infusion agar (*F35A*) and a poured plate with 0.4 ml. each, or substitute for the plate a 100-ml. French square bottle containing 15 ml. of melted agar. After inoculation the bottle is laid on its side and the agar allowed to harden. As a test for harmlessness, inoculate a guinea pig subcutaneously with 1.5 ml. of a 1:20 dilution of the concentrated vaccine. If the tests show contamination, retest. If contaminating microorganisms are still present, discard the material.

Dilution of Concentrated Suspension.—Dilute the bacterial suspensions within a week after preparation with cresolized salt solution so that each milliliter of typhoid vaccine contains 1000 million bacilli, 500 million of each strain. For typhoid-paratyphoid vaccines, dilute the suspensions so that each milliliter contains 500 million of each typhoid strain, 250 million of *S. paratyphi*-A and 250 million of *S. paratyphi*-B. The vaccines may be diluted before the completion of the tests for sterility and harmlessness provided they are satisfactory after three days.

From 50 to 60 liters or more of diluted typhoid vaccine are prepared at one time; from 10 to 15 liters of typhoid-paratyphoid vaccine. The salt solution for dilution is dispensed in 4-liter bottles. Determine the final volume to be contained in each bottle, usually about 3500 ml., by starting with a known volume of the concentrated vaccine of one strain. For example, assuming that the concentrated vaccine of strain No. 62 has a count of 35,000 million bacilli per milliliter, 50 ml. of concentrated vaccine should be diluted to 3500 ml., that is, $\frac{(35{,}000)\ (50\text{ ml.})}{500}$. Then determine the amount of concentrated vaccine of strain No. 40494 required if the count of this suspension is 50,000 million per milliliter.

$$\frac{(500)(3500\text{ ml.})}{50{,}000} = 35\text{ ml.}$$

Determine the amounts of cresolized salt solution and cresol required; e.g.

50 ml. + 35 ml. = 85 ml. = total volume of concentrated vaccine

3500 − 85 = 3415 = volume of cresolized salt solution required
3415 × 0.003 = 10.2 = cresol to be added to give 0.3-per-cent concentration in salt solution
3415 − 10.2 = 3404.8 ml. = volume of salt solution required

Before sterilization, the level of the salt solution in the bottles filled with the required volume (the nearest multiple of ten to the amount calculated) is marked by the lower edge of a strip of adhesive tape to gauge the loss from evaporation.

Assemble the required supplies in the room for rigidly aseptic technic. Set up a siphon equipped with a delivery tip protected by a glass bell in a 4-liter bottle containing distilled water. Dispense sufficient sterile distilled water into each bottle to bring the level of the solution to the lower edge of the tape and replace the cotton plug in each bottle of salt solution with a sterile rubber stopper. Add the preservative. Protect the stopper with cotton dampened in 1-per-cent cresol and shake each bottle vigorously until all of the cresol is in solution. Add the required volume of the concentrated suspensions and shake the bottles again to mix the contents thoroughly.

Before making sterility tests, remove about 5 ml. of vaccine from each bottle for comparison of turbidity. From 2 or 3 bottles, remove additional samples of about 10 ml. for the potency tests. For sterility tests, see p. 830. Cover the stoppers of the bottles with cotton dampened in 1-per-cent cresol, tie the stoppers down, cover with tin foil and paper, and store the bottles immediately in the cold room.

Control Tests of the Final Dilution.—As a control of the dilution of the vaccine, samples from every bottle are placed in standardized tubes (133 by 16 mm.) and compared for turbidity with samples from at least two previous lots of vaccine. If they do not agree closely, a count of the diluted vaccine is made by the Helber counting-chamber method. Seal two of the tubes with sterile rubber stoppers and store in the cold room for later use.

POTENCY TESTS

The potency of typhoid and typhoid-paratyphoid vaccines is determined by tests of their protective value for mice against virulent homologous strains. Mice are immunized by an intraperitoneal injection of vaccine and the degree of immunity induced is estimated from the survival rate following the intraperitoneal injection of live culture suspended in mucin. At present, there is no established control vaccine for use as a basis of comparison. As a culture control, the virulence of the test strain is determined in each test by injecting untreated mice with varying doses of culture.

The vaccines are tested against *Bact. typhosum*, strain No. 40494, and their potency is further controlled by occasional tests against freshly isolated or other heterologous typhoid strains. The procedures are similar to those employed at the National Institute of Health (3). Typhoid-paratyphoid vaccines should also be tested against *S. paratyphi*-A and -B strains. With these strains, it may be necessary to modify the procedures for the test. From time to time the agglutinogenic activity in rabbits of the typhoid-paratyphoid vaccines is also tested.

The selection of a control vaccine and methods for its preservation are under investigation. In addition, special studies are in progress to develop a standard procedure for determining the potency of vaccines based on the titration of the amount required to protect mice against a standardized dose of culture (4).

Immunization of Mice.—Inject 30 or more mice weighing from 14 to 16 grams intraperitoneally with 0.5 ml. of a 1:10 dilution in salt solution of the test vaccine. Use 2-ml. syringes and 24-gauge needles, three-eighths inch long. Immediately after injection, mark each mouse for identification. Set aside a group of 30 or more mice from the same stock and of the same weights for the controls on the virulence of the test strain. Mark these mice to distinguish them from the vaccinated animals. In both groups of mice, include 1 or 2 more than are required for the test to allow for the occasional deaths that occur, so that at least 10 mice will be available for each test dose of culture. Fourteen days later weigh all the mice. Divide the vaccinated and the control series each into groups of 10 mice so that the weights are approximately the same in each group and inject the test culture.

Test Culture.—Use a recent subculture of a dried preparation. Three or four days before the culture is to be used, add 0.5 ml. of broth to a dried preparation of the test strain and inoculate from the suspension a beef-infusion agar slant (*F34A*). Incubate the culture overnight. Inoculate with the growth a tube of double-sugar agar (*F50B*), streak a blood agar plate, and make a slide preparation stained by Gram's method. Store the agar slant in the cold room. If the culture appears typical and there is no evidence of contamination, on the day before the test make a transfer on a beef-infusion-agar slant and incubate it for from twelve to fourteen hours. Suspend the growth in 1 or 2 ml. of beef-infusion broth and make a slide preparation stained by Gram's method. Pass the suspension through a cotton filter, see p. 622, and dilute it with broth to equal in turbidity a glass standard previously matched to a suspension that contained 1000 million typhoid bacilli according to a direct count. Make 10-fold serial dilutions of the standardized culture as required. Make the dilutions that are to be used for injection in a 5-per-cent mucin preparation (see p. 702). From one or two of the dilutions for the virulence control test, make poured-plate counts. Pipette 0.5-ml. amounts of the dilutions into Petri plates in duplicate, then pour into each plate melted beef-infusion agar (*F35A*). Mix well by gently rotating the plates. Incubate for forty-eight hours and count the colonies.

Test Doses of Culture.—Inject the mice intraperitoneally with 0.5-ml. amounts of the selected culture dilutions. For vaccinated mice, the dilutions are usually 10^{-2}, 10^{-3}, and 10^{-4}, approximately 5 million, 500,000, and 50,000 bacilli; for the control mice, 10^{-6}, 10^{-7}, and 10^{-8}, approximately 500, 50, and 5 bacilli. Use 22- or 23-gauge needles one-half inch long.

Observation of Mice. Necropsies.—For inspection of mice, see p. 685. Autopsy all mice that die unless the number of deaths in a group exceeds six, then only six. Perform autopsies as soon as practicable and make cultures from the heart's blood on Endo agar (*F52A*). From one mouse in each group, inoculate also a tube of double-sugar agar.

Estimation of Potency.—At present, the potency of the test vaccine is estimated on the basis of the number of minimum lethal doses of culture against which mice are afforded protection. The M.L.D. of the test culture is considered to be the smallest number of bacilli that kills fifty per cent of a group of mice in seventy-two hours. As a rule the M.L.D. falls between 50 and 500 bacilli. Vaccinated mice that survive seventy-two hours after the injection of culture are considered protected. Fifty per cent or more of the vaccinated mice that receive 10,000 M.L.D. should survive.

PREPARATION FOR DISTRIBUTION

Typhoid vaccine is dispensed in 3- and 10-ml. amounts; typhoid-paratyphoid vaccine in 10-ml. amounts only. It is not released for distribution within less than three weeks from the date the suspension is heated, except in special emergencies. If kept under proper conditions, the vaccine is considered satisfactory for use for eighteen months from the date of preparation.

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CHAPTER 63

PREPARATION OF PERTUSSIS VACCINE

Pertussis vaccine is prepared for prophylactic use from selected phase-I strains of known antigenic value. Cultures grown on sheep-blood starch peptone agar are suspended in salt solution, centrifuged, and resuspended in fresh salt solution. The vaccine is sterilized by adding 'Merthiolate' which is adjusted in the final preparation to 0.01 per cent. The finished product contains 10,000 million bacilli per milliliter. The immunizing activity of the vaccine is determined by protection tests in mice. The methods for the preparation of the vaccine are, in general, similar to those developed at the Bureau of Laboratories, Michigan Department of Health.

The preparation of phase-I pertussis vaccine from cultures grown in a fluid medium, developed in this laboratory from Hornibrook's formula (1), is in progress. The available laboratory data indicate that such vaccines compare favorably with the present routine material. Because of the simpler technical procedures and the possible superiority of whole-fluid-culture vaccines that may contain soluble antigenic substances, the adoption of this method for the routine preparation of pertussis vaccine is under consideration. For a description of the method, see p. 767.

Strains

At present, two phase-I strains of *Hemophilus pertussis* are used. They were selected as tentative standard strains for vaccine production on the basis of their phase-I characters and antigenic activity in rabbits.

Collection No. 40103. Isolated from a cough plate taken March 14, 1940.
Collection No. 41405. Isolated from a cough plate taken November 4, 1941.

These strains were both isolated at this laboratory from cases of whooping cough about two weeks after onset.

Additional strains, including recently isolated cultures, are maintained for use in special tests and experimental studies. Comparative studies of the antigenic activity and valency of phase-I strains are continued with a view to the replacement, if indicated, of the strains used for vaccine production. Experience has shown the importance of selecting standard strains of marked stability and high antigenic activity for vaccine and serum production and also that if such strains are maintained under carefully controlled conditions, their essential biologic characters can be retained.

Maintenance of Strains.—The strains are maintained in the dry state. Prepare new dried preparations, as required, usually every one or two years. For vaccine cultures in active use, make subcultures from dried preparations every month and maintain the subcultures on slants of potato-infusion rabbit-blood agar, *F56B*, (175- by 25-mm. tubes) with transfers at weekly intervals. Discard the weekly transfers at the end of the month, after fresh subcultures have been made from a dried preparation. To a dried culture add 0.5 ml. of beef-infusion broth. Inoculate a slant and a plate of potato-infusion sheep- or rabbit-blood agar (*F56A*) with about 0.1 ml. of the culture suspension. Incubate the cultures at 34°–36°C. for forty-eight or seventy-two hours. When growth develops, make a slide preparation stained by Gram's method. If there is no evidence of contamination, inoculate two or three slants of rabbit-blood agar and incubate for forty-eight hours. If the cultures appear pure on examination, store them in the cold room and make fresh transfers after five days of storage.

Tests of Phase-I Characters (2).—The identity and purity of the cultures are controlled by the preliminary tests made in connection with each lot of vaccine. In addition, every six months, test fresh subcultures from dried preparations as follows. Note the morphologic and cultural properties and hemolytic activity on sheep-blood agar plates (*F56A*). Test for agglutination with phase-I serum, virulence for mice, and capacity to induce necrotic lesions on intracutaneous injection in rabbits (3).

Agglutination Tests.—The technic is that described on p. 19, except as indicated. Serum dilutions: Use the following dilutions of a phase-I serum—1:100, 1:500, 1:1000, 1:2000.

Limited production of antipertussis serum for therapeutic use is in progress. Trial bleedings and samples of whole bleedings from the rabbits under immunization with phase-I pertussis cultures are pooled for use as an aid in the identification of *H. pertussis*.

Bacterial Suspensions: Suspend in salt solution the growth from a 24- or 48-hour culture on rabbit-blood agar (*F56B*). Dilute the culture suspension to equal in density a glass standard previously prepared to match in turbidity a suspension of a 48-hour culture that contained 10,000 million bacilli per milliliter, according to a direct count.

The Test: As controls, use salt solution and normal rabbit serum diluted 1:100. Add 0.1 ml. of culture suspension to 0.1-ml. amounts of each serum dilution and the controls in 75- by 11-mm. tubes, shake vigorously for from three to five minutes, add 0.5 ml. of salt solution to each tube, read immediately, and record as indicated on p. 21. Definite agglutination should occur in the highest serum dilutions.

Virulence for Mice.—Inject mice of from 11 to 14 grams intraperitoneally with varying doses of a 24-hour culture in 5-per-cent mucin. Prepare the culture as for agglutination tests but suspend it in broth and make final dilutions in a mucin suspension. For preparation of mucin, see p. 702. Inject three mice with each dose of culture in 1-ml. amounts. At present, the following doses are used: approximately 25 million, 100 million, and 400 million bacilli. Two of three mice injected with 400 million or 100 million bacilli should die within ninety-six hours. Discharge mice that survive on the 8th day.

Intracutaneous Tests in Rabbits.—Prepare two rabbits, weighing from 3000 to 4000 grams each, by clipping the hair from lateral areas either side of the spine, inject intracutaneously 0.1-ml. amounts of varying dilutions of a salt solution suspension of a 24- or 48-hour culture. Dilutions containing approximately 500, 1000, and 2000 million bacilli are used. Make readings after twenty-four and forty-eight hours and seven days. Hemorrhagic reactions, developing into necrotic lesions, should be induced by the dilution containing 1000 million bacilli.

PREPARATION OF VACCINE

All procedures for the preparation of the vaccine are carried on in rooms designed for rigidly aseptic technic.

A convenient schedule provides for the preparation of two lots of concentrated vaccine a week, one with each of the vaccine strains. Work with the two strains is begun on different days. The starch-peptone agar with citrated sheep blood on which the cultures are grown is allowed to harden in one-liter Roux bottles laid on their sides. Usually a lot consists of from 120 to 150 bottles.

Supplies. Media: Starch-peptone agar with 20- or 25-per-cent citrated sheep blood (*F56.2*) in 140-ml. volumes in one-liter Pyrex Roux bottles with neck and cotton plug protected by a paper or muslin cap. The medium is inoculated within 2 weeks after its preparation.

Salt solution: Salt solution dispensed in 300- or 500-ml. Erlenmeyer flasks in 50-ml. amounts for planting and in 100-ml. amounts for rinsing harvesting outfits and for resuspending the centrifuged cultures; in 500-ml. flasks in 200-ml. amounts for final pooling of concentrated suspensions; in 4-liter Pyrex bottles in 3-liter amounts for washing off the growth from the agar cultures in Roux bottles; in 9-liter Pyrex bottles in the volumes required for diluting the vaccine.

'Merthiolate': A stock 1-per-cent solution of 'Merthiolate' and 1.4-per-cent sodium borate. A fresh solution is prepared each month.

Bottles and Flasks: Centrifuge bottles of 250 ml. containing about 5 grams of glass beads for collecting and centrifuging the culture suspensions; 500-ml. Erlenmeyer flasks for preliminary pooling of concentrated suspensions; graduated Pyrex 1-liter Blake bottles for final pooling of concentrated suspension; 9-liter Pyrex bottles for the diluted vaccines.

Pipettes: Graduated and bulb pipettes of different capacities in separate containers.

Siphons: Siphons equipped with bell-covered delivery tips for dispensing salt solution for washing off the growth from the agar cultures and for dispensing distilled water to replace the volume lost in sterilization of salt solution in bottles for dilution of the vaccines.

Special outfits: Outfits for closed systems in planting, final pooling of concentrated suspensions, and diluting the vaccines, and also special harvesting outfits are used. They are patterned after those used at the Bureau of Laboratories of the Michigan Department of Health. On the outfits the stoppers with connections are protected by gauze-covered cotton pads or, as required, by paper envelopes.

After use, submerge planting, harvesting, and final pooling outfits in water in covered containers for autoclaving. After sterilization, rinse the rubber tubing in running tap water and soak the glassware for at least one-half hour in a 0.3- to 0.5-per-cent hot solution of a soapless detergent (see p. 246). Rinse thoroughly in running water, dry in air, and reassemble. Replace used gauze filters. Wash closely-woven rayon filters, dry, and use again. Rinse in tap water siphons and outfits used for sterile solutions or suspensions, dry, and reassemble.

The bottles, pipettes, siphons, and outfits are sterilized the day before they are required; the salt solution within two or three days.

Preliminary Transfers. Identification and Purity Tests.—1st day. From a previously unopened culture of the most recent transfers of the vaccine strain, inoculate three slants of blood agar, *F56 B*, (175- by 25-mm. tubes), streak a sheep-blood agar plate (*F56 A*) and make a slide preparation stained by Gram's method for microscopic examination. Incubate the cultures at 34°–36°C. for forty-eight hours.

3d day. Examine the slants and plates. If the growth is typical and there is no evidence of contamination, test the growth from one of the slants for agglutination with phase-I serum. See "Tests of Phase-I Characters, Agglutination Tests." If typical agglutination is obtained, use the other two cultures for the inoculation of four Roux bottles. Pipette about 8 ml. of salt solution into each tube. Suspend the growth by scraping it from the surface of the slant with a loop. With a pipette inoculate two bottles with the suspension from one slant. Incubate the bottles for forty-eight or seventy-two hours.

5th day. Examine the bottles carefully. Discard any that appear to be contaminated. Pipette about 25 ml. of salt solution into each bottle in which the growth appears typical. Suspend the growth by tilting the bottle gently back and forth and make a slide preparation for microscopic examination. If the cultures and microscopic preparations appear satisfactory prepare the vaccine seeds by inoculating with a pipette four or five bottles from each suspension. The total number of vaccine seeds should be two more than 1/10 the number of bottles to be inoculated. Incubate the seeds for seventy-two hours.

Inoculation and Incubation of Cultures.—8th day. Examine the seed bottles carefully. Discard any that appear contaminated and make stained slide preparations from any in which contamination is suspected. Examine each bottle of agar to be inoculated. Use a lamp with a strong bulb and hold the bottle directly over the bulb to detect colonies in the agar. It is convenient to label the bottles at the time of examination, usually in the morning of the day the vaccine is to be planted. Use pipettes or a closed system for planting. (See Fig. 67.)

Connect a planting outfit to a seed bottle A and a 300-ml. Erlenmeyer flask B that contains 50 ml. of salt solution. Secure the stopper in the flask with a light cord tied around the neck of the flask and across the top of the stopper and wrap both stoppers with strips of cotton dampened in 1-per-cent cresol solution. Put a pinchcock on the rubber tubing that leads to the bell-covered delivery tip C. Hold the flask of salt solution in a horizontal position to keep the salt solution from the air-inlet tube, lower the seed bottle, and allow the salt solution to run from the flask into the bottle. Suspend the growth by tilting the bottle gently back and forth and then run the culture suspension back into the flask. Put a pinchcock on the rubber tubing leading to the seed bottle, invert the flask carefully so that the suspension does not run into the air-inlet tube and support it on a ring stand. Remove the pad protecting the delivery tip and dispense the suspension in about 5-ml. amounts into ten or twelve bottles.

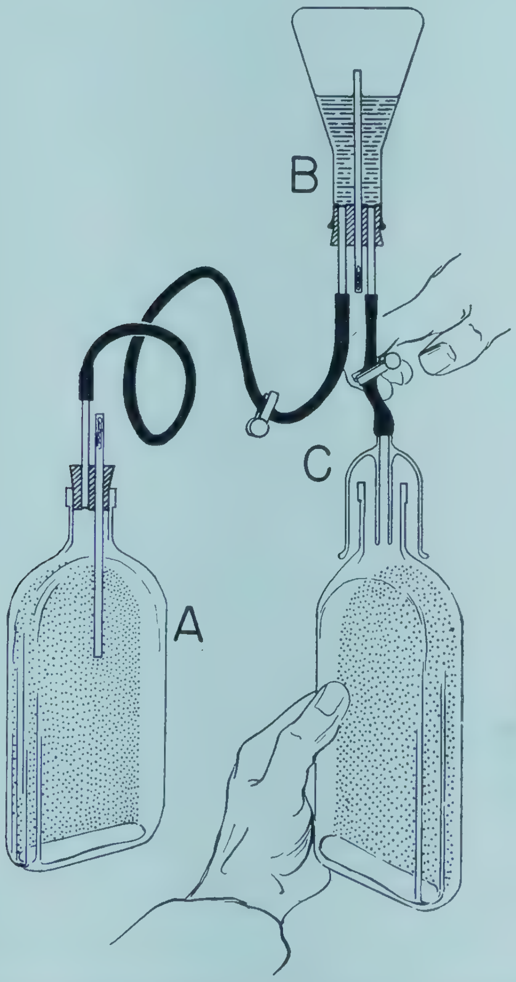

FIG. 67. OUTFIT FOR PLANTING *Hemophilus pertussis* FOR PREPARATION OF VACCINE

Use a fresh pipette or planting outfit for each seed bottle. Wash the inoculum over the surface of the agar. Incubate the cultures at 34°–36°C.

for from sixty-three to sixty-five hours. During the incubation period, tilt the bottles daily to spread the growth over the agar surface.

Harvesting of Growth. Centrifugation of Cultures.—11th day. Examine the bottles carefully and discard any that show definite contamination. Make stained slide preparations for microscopic examination from any in which contamination is suspected. Set up a siphon with a shielded delivery tip in a 4-liter bottle that contains two or three liters of salt solution. Dispense about 15 ml. of salt solution into each bottle to wash off the growth. By means of a harvesting outfit (see Fig. 68) attached to the vacuum line V, filter the suspensions from 10 or 12 Roux bottles through six thicknesses of gauze A and collect them in a 250-ml. centrifuge bottle B containing glass beads. Avoid drawing air through the outfit when transferring the pipette C from bottle to bottle by using a pinchcock on the rubber tubing. For rinsing the outfits use 100-ml. amounts of salt solution dispensed in 300-ml. Erlenmeyer flasks. Insert the pipette of the outfit into a flask and draw about 100 ml. of the solution through the outfit adding it to the suspension in the centrifuge bottle. Replace the stopper with connections in the centrifuge bottle with a sterile cotton plug. Use a fresh harvesting outfit, centrifuge bottle, and flask of salt solution for every group of 10 or 12 Roux bottles. From each lot of pooled culture suspension, pipette about 0.1 ml. on a sheep-blood agar plate, streak with a loop, and make a slide preparation. Centrifuge the suspensions in a refrigerated

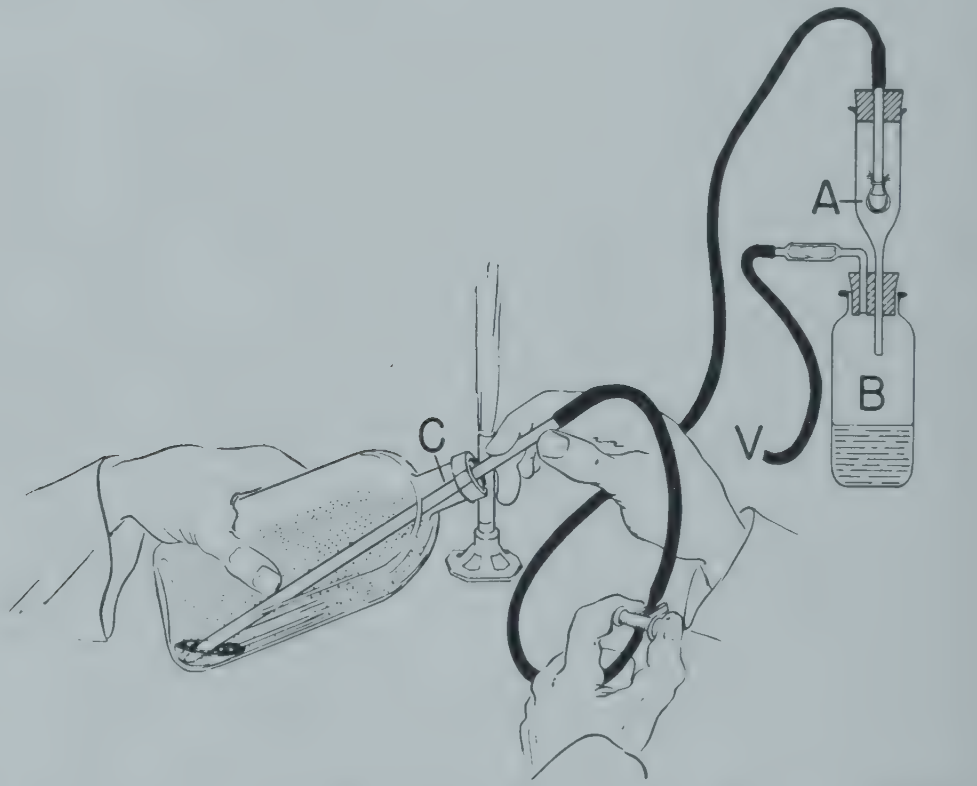

Fig. 68. Outfit for Harvesting Suspensions of *Hemophilus pertussis*

centrifuge at 2500 r.p.m. for one and one-half hours. Draw off the supernatant by vacuum. For this purpose, it is convenient to use a 4-liter bottle fitted with a 2-holed rubber stopper with glass and rubber tubing connections, one for the vacuum and the other for a 10-ml. unplugged pipette through which the supernatant is drawn. Take a fresh pipette for each centrifuge bottle. To resuspend the cells, add to each cup about 20 ml. of salt solution that contains 0.02 per cent of 'Merthiolate'. Replace the cotton plugs in the centrifuge bottles with rubber stoppers and shake vigorously. Store the suspensions in the cold room.

Preliminary Pooling of Concentrated Suspensions.—After two or three days, repeat the purity tests on the suspensions in the centrifuge bottles. From each, make a stained slide preparation for microscopic examination, streak a blood-agar plate, and, in addition, inoculate two tubes of Hitchens's medium that contains 0.05-per-cent sodium thioglycollate (*F32B*), one with 0.5 ml. and one with 0.1 ml. of suspension. Incubate the plates and Hitchens's medium for seven days. Pool in 500-ml. Erlenmeyer flasks the suspensions that show no evidence of contamination. Use harvesting outfits and pool suspensions from four or five centrifuge bottles in each flask. Rinse the centrifuge bottles with salt solution containing 0.02 per cent 'Merthiolate' and add the rinsings to the pool. From each pool, take samples for preliminary turbidity determinations and inoculate two tubes each of aerobic and anaerobic "sterility-test" broth (*F3B*) with 0.25 ml. of the concentrated vaccine, and one tube of Hitchens's sodium thioglycollate medium with 0.5 ml. In addition, test the viability of the pertussis bacilli by streaking a blood-agar plate (*F56A*). Return the pooled concentrated suspensions to the cold room and store for at least seven days.

Preliminary Determinations of Bacterial Concentration.—Preliminary determinations of the approximate bacterial count of concentrated suspensions are made by comparing the turbidity of various dilutions with that of a glass standard. A standard for each vaccine strain is prepared to equal in turbidity a freshly-harvested vaccine suspension containing 10,000 million bacilli per milliliter according to the Helber counting-chamber method. For bacterial count, see p. 752. To determine the density of a concentrated suspension in relation to the control, use previously standardized tubes (133- by 16-mm.). Pipette 0.5 ml. of the vaccine into each of three or four tubes. Add salt solution to one tube until the suspension approximates the density of the control and to the others so that the concentrations are slightly higher or lower than that in the first tube. The consecutive dilutions in a series are usually 1:10, 1:11, 1:12, etc. Select the dilution that most closely resembles the control in density. If the dilution selected is 1:13, the concentrated suspension contains approximately 130,000 million bacilli per milliliter.

Final Pooling of Concentrated Suspensions.—On the basis of the preliminary determinations of the bacterial concentration, pool concentrated suspensions of each strain separately in 1-liter Pyrex, graduated Blake bottles so that each final pool will be sufficient for 20 or 30 liters of diluted vaccine. Use a closed system (see Fig. 69) with three connections, one for the flask of concentrated suspension A, one for the graduated Blake bottle B, and the third for a 500-ml. Erlenmeyer flask C that contains 200 ml. of salt solution to which 'Merthiolate' has been added to give a 0.02 per cent concentration. Siphon the concentrated suspension from the flask into the bottle through a closely-woven rayon filter D, run salt solution into flask A to rinse it, and draw the rinsings into the bottle. Transfer the siphon to another flask of concentrated suspensions and repeat. Usually concentrated suspensions from two or three flasks are pooled in each bottle. Replace the stopper with connections in the Blake bottle with a rubber stopper. Shake vigorously and remove a 5-ml. sample for determinations of the bacterial count. Repeat the sterility and viability tests as for preliminary pools of concentrated vaccines, make a preliminary determination of the bacterial count, and inject a guinea pig subcutaneously

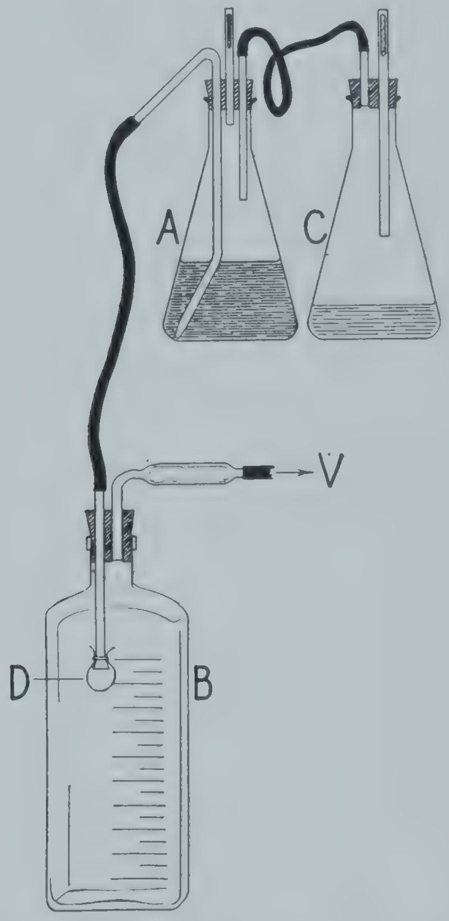

FIG. 69. OUTFIT FOR FINAL POOLING OF CONCENTRATED SUSPENSIONS OF *Hemophilus pertussis*

with 1.5 ml. of a dilution containing approximately 20,000 million bacilli per milliliter.

Final Determinations of Bacterial Concentration.—Final standardization of the vaccine is at present based on the Kjeldahl nitrogen content.

There is usually a lapse of several weeks between harvesting vaccine and the pooling of concentrated lots on which final standardization is done. Since some changes in the turbidity of vaccine suspensions on storage, as determined by measurements with a photoelectric turbidimeter, have been observed, the nitrogen content appears to offer a better basis for preparing diluted vaccines of uniform concentration.

The average nitrogen content of 1-ml. amounts of suspensions containing 10,000 million bacilli was determined for each vaccine strain. Each of ten freshly harvested suspensions of each strain was filtered through cotton, counted by the Helber counting-chamber method and diluted to contain 10,000 million bacilli per milliliter. The average total nitrogen content of cells and supernatant per milliliter was found to be 0.0406 ± 0.0024 mg. for strain No. 40103, 0.0391 ± 0.0036 mg. for strain No. 41405, the precision measure being the standard deviation of the mean. As a check on these established standards of nitrogen content, at intervals of four or six months, samples of freshly harvested vaccines should be counted, diluted to 10,000 million bacilli per ml., and the nitrogen content determined.

Shake vigorously the sample of vaccine taken after final pooling of the concentrated suspensions. Determine the nitrogen content per milliliter. See p. 121. To determine the bacterial count, divide the calculated amount of nitrogen per milliliter by the amount that represents 10,000 million bacilli per milliliter and multiply by 10,000 million.

Dilution of Concentrated Suspension.—Dilute the concentrated vaccines so that each milliliter contains 10,000 million bacilli, 5000 million of each of the two strains. Add 'Merthiolate' to give a final concentration of 0.01 per cent.

From 20 to 30 liters of the diluted material are prepared at one time. If some concentrated suspension remains it may be stored in the cold room and used, as required, in later diluted lots. The salt solution for dilution is dispensed in 9-liter Pyrex bottles. Determine the final volume to be contained in each bottle, usually from 6 to 8 liters, by starting with a known volume of the concentrated vaccine of one strain. The calculations and procedures are similar to those for the dilution of typhoid vaccine, p. 753, except as indicated.

Add 'Merthiolate' in a 1-per-cent solution to give a final concentration of 0.01 per cent. For example, assuming that a total of 600 ml. of concentrated vaccine is to be diluted and that 8000 ml. is the final volume, 68 ml. of the 'Merthiolate' solution should be added; that is,

8000 × 0.01% = 0.8 gm. = total amount of 'Merthiolate' required.
600 × 0.02% = 0.12 gm. = amount of 'Merthiolate' previously added to concentrated vaccine.

0.8 − 0.12 = 0.68 gm. or 68 ml. of a 1-per-cent solution of 'Merthiolate' to be added.

Add to the salt solution the calculated volumes of the concentrated suspensions and 5-per-cent excess to replace any loss from filtration, by siphoning them from the graduated Blake bottles into the 9-liter bottles. The suspensions are filtered through small rayon bags secured to the opening of the glass-inlet tube in the 9-liter bottles. Use a different outfit for each concentrated vaccine. From 2 or 3 bottles of the diluted vaccine, remove 15-ml. samples and pool for potency tests.

Control Tests of the Final Dilution.—As a control of the dilution of the vaccine, samples from every bottle are placed in standardized tubes (133- by 16-mm.) and compared for turbidity with samples from at least two previous lots of vaccine. If they do not agree closely, a determination of the nitrogen content is made. Seal one of the tubes with a sterile rubber stopper and store in the cold room for later use.

POTENCY TESTS

Mice are immunized by intraperitoneal injections of vaccine and the degree of immunity induced is determined by the survival rate following intracerebral injection of live culture (4). In each test, as a basis of comparison in estimating the potency of the test vaccine, mice are immunized with a vaccine tentatively selected as a control. The virulence of the test culture is determined in each test by injecting untreated mice with varying doses of culture. The minimum lethal dose is considered to be the smallest number of bacilli that kills three or more of a group of five mice of from 11 to 14 grams in fourteen days.

At present, groups of mice of from 8 to 11 grams are injected intraperitoneally with 0.5-ml. amounts of suitable dilutions of vaccine. The total dosage of vaccine for the different groups of mice immunized ranges from 1000 million bacilli with 2- or 4-fold decrements to 62.5 million bacili. At least ten mice are injected with each dose of vaccine. On the day immunization is begun, a group of mice from the same stock and of the same weights as the vaccinated mice, is set aside for virulence controls. Seven days after the vaccine dose, all the mice are injected intracerebrally under ether anesthesia with a live culture. At present, a strain heterologous to the vaccine is used. It is usually fatal to mice in doses of 500 bacilli or less. The test culture is grown for twenty-four hours on rabbit-blood agar (*F56B*), suspended and diluted in a 1-per-cent solution of casamino acids to equal in density a glass standard of turbidity representing 10,000 million bacilli per milliliter. A slide preparation stained by Gram's method is examined. Further serial dilutions of the standardized culture suspension are made as required. The test doses of culture, which may vary with the test strain and the immunizing doses of vaccine, are injected in 0.04-ml. amounts. Usually the test dose for vaccinated mice contains approximately 5 million bacilli. For the control mice, doses of approximately 2000, 400, and 80 bacilli are used and five mice are injected with each dose. Injections should be completed within one hour from the time the culture is diluted. For the technic of intracerebral injections, see p. 80. For anesthetization of mice, see p. 74.

Work is in progress to establish a standard procedure for the potency tests. The effect of different routes of administration, of varying the number of injections and dosage of vaccine, the test dose of culture, and the intervals between vaccine and culture injections are being studied.

The mice are observed for fourteen days after the injection of the test culture. Most of the deaths occur between the 3d and 10th days. Deaths that occur within forty-eight hours are omitted from the calculations for the estimation of potency since they are usually due to injury at the time of injection. Autopsies are performed as soon as practicable and sheep-blood agar plates (*F56A*) are inoculated with cultures from the brain.

No minimum standard of immunizing potency has as yet been established. The test vaccine should approximate the control vaccine which, under the present conditions of the test in an immunizing dosage of from 200 to 500 million bacilli, usually protects at least 50 per cent of the mice against from 1000 to 5000 M.L.D. of culture.

PREPARATION FOR DISTRIBUTION

Pertussis vaccine is dispensed in 10- and 20-ml. amounts. If kept under proper conditions, the final preparation is considered satisfactory for use for eighteen months from the earliest date of harvesting.

PREPARATION OF WHOLE-FLUID-CULTURE VACCINE

The strains used, the procedures for their maintenance, and the tests of phase-I characters are the same as those for vaccine prepared from cultures grown on sheep-blood agar.

The fluid-medium vaccine is prepared from cultures grown for seventy-two hours in casein-hydrolysate medium with yeast dialysate (*F29.2*) (5). Work with the two strains is begun on different days. Usually a lot consists of from fifty to seventy-five or more bottles.

Supplies. Media: Casein-hydrolysate medium with yeast dialysate dispensed, at present, in 200-ml. volumes in 1-liter Pyrex Roux bottles with neck and cotton plug protected by a paper or muslin cap, and in 6-ml. volumes in 150- by 19-mm. tubes.

Salt solution: Salt solution dispensed in 3- or 4-liter amounts in graduated 4-liter Pyrex bottles for diluting the vaccine.

'Merthiolate': A stock 1-per-cent solution of 'Merthiolate' and 1.4-per-cent sodium borate. A fresh solution is prepared each month.

Bottles: Graduated 4-liter Pyrex bottles for collecting the culture suspensions; 500-ml. centrifuge bottles for centrifuging portions of the preliminary pools of vaccine if the bacterial count is less than 10,000 million bacilli per milliliter; 9-liter Pyrex bottles for pooling the standardized suspensions of the two strains.

Special outfits: Outfits for closed systems in planting and in preliminary and final pooling of vaccine are used. For the care of these outfits, see p. 760.

The planting outfit consists of a two-holed rubber stopper, for insertion into the vaccine seed bottle, with an air-inlet tube and a glass and rubber tubing connection to a bell-covered delivery tip.

The preliminary pooling, or harvesting, outfit has a two-holed rubber stopper that fits the vaccine culture bottles, with an air-inlet tube and glass and rubber tubing connections to a second two-holed rubber stopper, also equipped with a cotton guard, which is inserted into the 4-liter collection bottle.

The outfit for diluting and final pooling of the vaccine is similar to that described for a closed system of siphoning, p. 621.

Preliminary Transfers. Identification and Purity Tests.—1st day. From a previously unopened culture of the most recent transfers of the vaccine strain, inoculate a slant of potato-infusion rabbit-blood agar, *F56 B*, (175- by 25-mm. tube), streak a sheep-blood agar plate (*F56 A*), and make a slide preparation stained by Gram's method. Incubate the slant for twenty-four hours, the plate for five days.

2d day. Examine the slant and plate. If there is no evidence of contamination, inoculate one tube of fluid medium. Incubate in a slanted position for forty-eight hours.

4th day. Transfer 0.5 ml. of the fluid culture to a fresh tube of medium. Incubate for forty-eight hours.

6th day. Inoculate each of the tubes of fluid medium, one more than the number of seed bottles required, with 0.5 ml. of culture. Incubate seventy-two hours.

9th day. Make microscopic preparations stained by Gram's method from each tube of culture and examine. Test the agglutination reactions of the culture in one tube with phase-I serum (see p. 758). If the morphologic and serologic characters are typical, use the remaining cultures for the inoculation of fluid medium in Roux bottles to be used for seeds. Pipette the contents of one tube into each bottle. Inoculate at least one bottle more than one-tenth of the number to be planted for vaccine. Incubate the bottles, laid on their sides to give maximum surface area to volume ratio, for seventy-two hours.

12th day. Make microscopic preparations from each seed bottle of culture. Discard any in which contamination is noted or suspected. Use a closed system for planting.

Connect a planting outfit to a well-shaken seed bottle, secure the stopper in the bottle with a light cord tied around the neck of the bottle, and wrap the stopper with a strip of cotton moistened with 1-per-cent cresol solution. Put a pinchcock on the rubber tubing that leads to the bell-covered delivery tip. Invert the seed bottle carefully so that the culture does not run into the air-inlet tube and support the bottle on a ring stand.

Dispense the culture, in about 15-ml. amounts, into ten or twelve bottles. Use a fresh planting outfit for each seed bottle. Incubate the cultures at 34° to 36°C. for seventy-two hours. Shake each bottle by hand once a day.

Harvesting of Culture.—15th day. Examine the bottles carefully and discard any that show definite contamination. Make stained slide preparations for microscopic examination from any in which contamination is suspected and from at least one from each group inoculated with the same seed. If the cultures appear to be pure and typical, pool them in groups of ten or twelve to give from two to two and one-half liters of culture in graduated 4-liter Pyrex bottles.

Connect a harvesting outfit to a graduated 4-liter bottle and to a vaccine culture bottle. Protect both stoppers with cotton strips dampened in 1-per-cent cresol solution. Put a pinchcock on the rubber tubing and invert the culture bottle. Support it in a rack and allow the culture to run into the collection bottle. Replace the culture bottle and repeat. It is convenient to use a rack that holds two or more culture bottles so that more than one pool can be made at one time.

Use a fresh harvesting outfit for each pool. From each bottle of pooled culture, with a pipette, remove a sample, streak a sheep-blood agar plate with about 0.1 ml., and make a slide preparation. Record the volume of culture in each bottle and add 'Merthiolate' in a 1-per-cent solution to give a final concentration of 0.01 per cent. After the addition of preservative, pipette into vials samples of about 8 ml. from each pool for determinations of the bacterial count. Store the vaccine in the cold room. After three or four days' storage, test each bottle of vaccine for freedom from contamination, using 2 ml. to inoculate sterility-test broth and 6 ml. for Hitchens's medium (p. 830, and streak a sheep-blood agar plate. No growth should occur in the sterility-test media. *H. pertussis* in pure culture should develop on the plates.

Determination of Bacterial Concentration.—At present, the bacterial count of a sample from one bottle of pooled culture of a lot is determined by the Helber counting-chamber method on the day of harvesting. This sample is then diluted to contain 10,000 million bacilli per milliliter and serves as a turbidity standard in estimating the bacterial concentration of the other pools of the lot. For the turbidity determinations, see p. 763.

Dilution of Concentration of Vaccine and Final Pooling.—From ten days to two weeks after harvesting, adjust the bacterial concentration in each preliminary pool of vaccine so that 1 ml. contains 10,000 million bacilli per milliliter. Pool equal amounts of standardized vaccine of the two strains.

If any preliminary pools of vaccine remain, they may be stored in the cold room and used with other lots prepared within a month.

The salt solution for dilution is dispensed in specified volumes in 4-liter bottles. Determine the amount required for each bottle of vaccine. Add 'Merthiolate' to the salt solution to give a final concentration of 0.01 per cent and by means of a closed system of siphoning add the required amount of salt solution to the vaccine in each graduated 4-liter bottle. If the bacterial count of a preliminary pool of vaccine is less than 10,000 million bacilli per milliliter, calculate the reduction in volume necessary. Transfer a portion of the vaccine, usually from 200 to 500 ml., to a centrifuge bottle, centrifuge, and discard the calculated amount of the supernatant. Resuspend the cells in the remaining supernatant by drawing both up in a pipette and expelling them several times. Add the suspension to the pool.

After the bacterial count of each preliminary pool has been adjusted, pool an equal volume, usually from three to four liters, of vaccine of each of the two strains in 9-liter Pyrex bottles by means of a closed system of siphoning. Use a different outfit for each vaccine strain and additional outfits if a final lot consists of more than three bottles. From each bottle of the final preparation, remove 10-ml. samples and pool them for potency tests and for a test of harmlessness in a guinea pig. Inject the guinea pig with 2.0 ml. subcutaneously. Inoculate sterility-test media and a sheep-blood agar plate with vaccine from each bottle as for preliminary pools. Store the vaccine in the cold room. If *H. pertussis* develops on the plates, repeat the viability tests after a week, using the samples taken for control tests of the bacterial concentration. Usually no growth occurs if the vaccine has been stored for from two to three weeks after treatment with 'Merthiolate'.

Control Tests of the Adjustment of Bacterial Count and Final Pooling.—As a control of the bacterial concentration, samples from every bottle of the final preparation are placed in corked standardized tubes (150- by 16-mm.) and compared for turbidity with samples from at least two previous lots. If they do not agree closely, a direct count is made. Store the samples in the cold room until the viability tests are completed, then seal one of the tubes with a sterile rubber stopper and keep for later use.

POTENCY TESTS. PREPARATION FOR DISTRIBUTION

The procedures are the same as for vaccine prepared from cultures grown on sheep-blood agar (see p. 766), except that a final viability test is made on a vial of the dispensed vaccine.

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CHAPTER 64

PREPARATION OF BRUCELLA ABORTUS VACCINE

Vaccine for use in the treatment of suitable cases of undulant fever is prepared for limited distribution from a stock strain of *Brucella abortus* (1, 2, 3). The vaccine is a heat-killed cresolized suspension containing 2000 million bacilli per milliliter. The procedures for its preparation are the same as those for typhoid vaccine except as indicated.

Maintenance of Strain

The strain in use, Collection No. 322, was isolated in 1929 at this laboratory from the blood of a human case of undulant fever. It is maintained in the dry state. Prepare dried cultures as required, usually every one or two years.

PREPARATION OF VACCINE

Special precautions are observed in handling live cultures of *Br. abortus*. See "General Bacteriologic Technic," p. 4.

Preliminary Transfers. Identification and Purity Tests.—To a dried culture, add 0.5 ml. of beef-infusion broth and inoculate with the suspension a beef-infusion-agar slant (*F34A*). Incubate the culture for twenty-four or forty-eight hours. When growth develops, transfer it to a beef-infusion-agar slant, streak a blood-agar plate, and make a slide preparation stained by Gram's method. Incubate the cultures for forty-eight hours. If growth is typical and there is no evidence of contamination, proceed with the preparation of the preliminary vaccine cultures. From the most recent transfer on beef-infusion agar prepare one beef-infusion-agar culture (133- by 16-mm. tube) for each flask to be inoculated and incubate the cultures for forty-eight hours. Add 2 ml. of salt solution to each slant and make a slide preparation.

Inoculation and Incubation of Cultures.—Usually from 4 to 6 Roux bottles of 2-per-cent beef-infusion agar are inoculated in a lot, each bottle with the suspension from one seed tube. Incubate for forty-eight hours.

Harvesting of Growth. Heating of Suspension. Sterility Tests. Bacterial Count of Vaccine.—Pool the suspensions from the flasks in a graduated 200-ml. French square bottle and heat at 60°C. for one hour. No growth should develop on the blood-agar plate streaked with the heated suspension. When the sample of the heated and cresolized suspension is removed for sterility tests, take an additional 2 ml. for the bacterial count.

Store the sample used for the sterility tests in the cold room. The bacterial count is usually made from three to five days later if the tests indicate that the vaccine is sterile. Dilute the concentrated suspension so that each milliliter contains 2000 million bacilli.

Test the sample of the concentrated suspension with *Br. abortus* agglutinating serum. Dilute the suspension to the density of glass standard No. 3, then test with the serum, usually in dilutions of 1:100, 1:200, 1:400, and 1:800. Incubate the mixtures at 48°–52°C. for four hours, place in the cold room, and make readings the following morning.

PREPARATION FOR DISTRIBUTION

Br. abortus vaccine is dispensed in 6-ml. amounts. Material more than six months old is not distributed.

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CHAPTER 65

PREPARATION OF SODIUM CITRATE SOLUTION

A supply of 5-per-cent sodium citrate solution for use in the collection of parent's whole blood for the modification or prevention of measles is maintained for distribution. One hundred grams of sodium citrate C.P. ($Na_3C_6H_5O_7 \cdot 2H_2O$) are dissolved in distilled water and made up to a volume of 2000 ml. with distilled water. The solution is sterilized by steam at 121°C. for thirty minutes. The sterility tests of the material in bulk and in final containers are similar to those for other products except that the mouse receives 1 ml. of a 1:15 dilution of the 5-per-cent solution. The material is dispensed for distribution in 2.5-ml. amounts contained in 5-ml. bottles.

CHAPTER 66

PREPARATION OF NORMAL HORSE SERUM

Normal horse serum is prepared chiefly for the treatment of hemophilia. The serum is obtained from a healthy horse, mallein tested, and previously immunized with tetanus toxoid, and which has never been used for the production of immune sera. The horse must have been under observation for at least two months and have a record of normal temperatures preceding the bleeding. After the serum has been drawn off from the clot and the preliminary sterility tests made, 10-per-cent 'Merthiolate' to give a final concentration of 0.01 per cent is added as preservative. The serum is filtered, tested for sterility, then dispensed in 20-ml. volumes, and the usual tests for sterility and harmlessness are made. If kept under proper conditions, it is considered satisfactory for use for eighteen months from the date of the bleeding.

CHAPTER 67

PREPARATION OF OLD TUBERCULIN (KOCH'S O.T.)

Tuberculin is prepared for intracutaneous or cutaneous tests to demonstrate the reactivity that is associated with the presence of a tuberculous infection. It consists of the sterile filtrate of a heated 5-per-cent glycerol broth culture of the human tubercle bacillus concentrated to one-tenth of its original volume. The finished product is tested clinically before it is released for distribution.

Maintenance of Strain.—A human strain of *Mycobacterium tuberculosis*, Collection No. 34238, is used for the preparation of tuberculin. It is maintained on 5-per-cent glycerol beef-infusion broth and transferred at intervals of from three to four weeks.

Preliminary Culture. Purity Tests.—Use a culture that has been incubated on 5-per-cent glycerol broth (*F8*) at 36°–38°C. for from three to four weeks or until the growth covers the surface of the medium and is sufficiently heavy to permit satisfactory transfer of the pellicle with a large nichrome wire loop. In removing the culture from the incubator, take care that the pellicle does not sink to the bottom or become moist on the surface. Examine for evidence of contamination; the culture should show the characteristic pellicle with clear broth below. Make a slide preparation stained with carbolfuchsin and alkaline methylene blue. Inoculate a tube each of aerobic and anaerobic "sterility test broth" (*F3B*), and streak a 0.2-per-cent glucose beef-infusion agar plate (*F35A*). No growth should develop.

Inoculation of Medium. Incubation. Purity Tests. Heating of Culture.—Inoculate the medium, contained in 1-liter flat bottles, by floating upon the surface small portions of the pellicle from the seed culture. To prevent evaporation cap the bottles with tin foil. Incubate at 36°–38°C. for from six to nine weeks depending upon the rate of growth. Examine the contents in each bottle and discard bottles that are contaminated. Heat the cultures in steam at 100°C. for one hour.

Concentration of Heated Culture.—The following day, pass the heated cultures through soft filter paper. Pour the measured volume of broth filtrate into one or more weighed evaporating dishes. Reweigh and place over a steam bath. Evaporate under a hood with a good draft. When the weight has reached approximately one-tenth that of the original, pour the concentrated material into a graduated cylinder. After cooling, make up the volume if necessary with distilled water to equal one-tenth that of the original broth filtrate. Transfer to a bottle fitted with a stopper. Before the final cleaning, rinse thoroughly in hot water all glassware used in the preparation of the concentrated material, since it is exceptionally heat-stable and adherent.

Addition of Preservative. Filtration. Sterility Tests.—Add cresol to give a final 0.3-per-cent concentration, filter promptly, then make sterility tests. Inject 0.5 ml. subcutaneously into the inguinal region of a guinea pig. After six weeks, chloroform the animal and make post-mortem examination to assure the absence of tuberculous lesions.

POTENCY TESTS

The activity of each lot of tuberculin is tested clinically by the intracutaneous method (Mantoux) on a series of tuberculous patients. Separate syringes and needles are reserved for the purpose. After use, boil in soap solution for ten minutes. Use a standard tuberculin as a control. For the test, dilute the tuberculin with freshly prepared salt solution. For children under eight years, use 0.1 ml. of a 1:10,000 dilution; for older children and adults, 0.1 ml. of a 1:1000 dilution. No lot is released unless it compares favorably with the preparation used as a standard.

The intracutaneous method for testing the potency of tuberculin on tuberculous guinea pigs has also been employed to a limited extent.

PREPARATION FOR DISTRIBUTION

Tuberculin is dispensed in 0.5-ml. volumes. If kept under proper conditions, the material is considered satisfactory for use for three years.

CHAPTER 68

PREPARATION OF DILUTED DIPHTHERIA TOXIN FOR THE INTRACUTANEOUS TEST OF SUSCEPTIBILITY (SCHICK)

A stable dilution of diphtheria toxin containing one-fiftieth of a minimum lethal dose in 0.1 ml. is prepared with sodium chloride solution buffered with protone (1) for use in the intracutaneous test of susceptibility (Schick). A similar dilution of heated toxin is prepared for the control injection.

SELECTION OF TOXIN

A diphtheria toxin at least two years old accurately standardized, and containing 250 M.L.D. or more per milliliter, is selected. In order that a stabilized toxin of the required potency may be available when needed, it is advisable to set aside at the time of preparation portions of different lots that contain 500 or more M.L.D. per milliliter. Tests are made from time to time to ascertain the degree of stability and to form a basis for selection.

Toxins prepared in veal infusion broth (*F12*) have been used for dilution. The stability of diluted toxins prepared in infusion-free medium (*F20*) is under investigation.

Standardization of Toxin

Toxins reserved for dilution are standardized in guinea pigs weighing from 250 to 280 grams, see "Production and Standardization of Diphtheria Toxin," p. 626. In the preliminary test 3 doses, such as 0.004, 0.003, and 0.002 ml., are injected and two or more animals used for each. Later tests based on these results are made at closer intervals, such as 0.0035, 0.003, and 0.0025 ml. In the final test 5 animals are injected with the estimated minimal lethal dose. At least 4 should die in somewhat less than ninety-six hours showing lesions typical of uncomplicated diphtheria poisoning.

When the M.L.D. has been determined, at least 2 guinea pigs are injected intracutaneously as a preliminary test with 1/50, 1/250, and 1/500 of an M.L.D., each contained in 0.1 ml. Within a week, the 1/50 M.L.D. should induce marked necrosis, the 1/500, a reaction corresponding to that designated as definitely positive in a person. At the same time, each animal is injected with 0.1 ml. of similar dilutions of the heated toxin. Any very slight initial reaction should disappear after twenty-four hours. If found satisfactory in animal tests, the toxin is tested intracutaneously on persons using 1/50 M.L.D. contained in 0.1 ml. Five susceptible (preferably of different degrees of susceptibility) and 5 insusceptible persons are selected for the tests. If the toxin fails to give characteristic reactions, as happens rarely, it should be rejected for the intracutaneous test.

PREPARATION OF TOXIN DILUTION

Diluent.—Sodium chloride solution buffered with protone in a 0.05-per-cent concentration (*F60*), from pH 8.0 to 8.4 is used as diluent.

Procedure.—Remove from the stock bottle of the selected toxin a small amount sufficient for the preparation of the diluted material. Protect from the light, and allow to come to room temperature. Siphon the required amount of diluent that has come to room temperature into graduated bottles (usually 7-liter lots are prepared). For preparation of the siphon, see "General Instructions," p. 620. Add 'Merthiolate' in a 10-per-cent solution to give a final concentration of 0.005 per cent. Pipette the calculated amount of toxin to give a dilution containing 1/50 M.L.D. per milliliter. After each addition mix thoroughly by rotating the bottle. Pass through a Mandler filter candle, discard the first 500 ml. filtered, and stop the filtration before the inner glass cylinder starts to empty. Take two samples from each bottle; one for an immediate test of potency (keep at room temperature protected from the light), the other to be incubated at 36°C. for forty-eight hours for a subsequent potency test to determine stability. Make the required sterility tests. Store in the cold room, protected from the light.

Heat a sufficient amount of undiluted toxin in a water bath at 70°–75°C. for thirty minutes, and cool to room temperature. Prepare the same amount of diluted heated toxin as of the unheated. Pipette into the bottles of measured diluent, to which 'Merthiolate' has been added, a volume of the heated toxin equal to that of the unheated toxin used for the toxin dilution. Rotate to mix thoroughly. Filter and make the required sterility tests. Save the remainder of the undiluted heated toxin for an animal test of detoxification.

STANDARDIZATION OF TOXIN DILUTION

Potency tests are made immediately after filtration by injecting each of from 3 to 5 guinea pigs with 5 ml. (1 M.L.D.) of the toxin dilution, see p. 626. The stability of the material is determined by a similar test of the incubated sample. The animals should die in accordance with requirements for the final standardization of the undiluted toxin. Occasionally, confirmatory tests are made on persons.

As a test of detoxification of the heated toxin dilution, each of 2 guinea pigs is injected subcutaneously with 1 ml. of the undiluted heated toxin and 2 intracutaneously with 0.1 ml. of the diluted material. The symptoms in the former should be comparatively mild and the reactions in the latter absent or very slight, disappearing after twenty-four hours.

Any test in which irregular results are obtained is repeated. If the results of the tests are not satisfactory, the material is discarded.

PREPARATION FOR DISTRIBUTION

When the potency tests are completed the diluted toxin and heated toxin are dispensed in 2- and 5-ml. volumes. If kept under proper conditions, the material is considered satisfactory for use for one year from the date of the satisfactory potency test following dilution. The date is not extended beyond this time; any material remaining is discarded.

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CHAPTER 69

PREPARATION OF DILUTED STREPTOCOCCUS TOXIN FOR THE INTRACUTANEOUS TEST OF SUSCEPTIBILITY

A stable dilution of standard streptococcus toxin containing one skin test dose in 0.1 ml. is prepared with borate-buffered sodium chloride solution (1) for use in the intracutaneous test of susceptibility of persons to the toxin. A similar dilution is heated for the control injection.

PREPARATION OF TOXIN DILUTION

Toxin.—A stable toxin, strain No. 165, aged for six months or longer, accurately standardized, and containing at least 40,000 skin test doses (S.T.D.) per milliliter is selected. In intracutaneous tests the toxin should have induced a reaction that persisted for at least twenty-four hours in susceptible persons, and no reaction should have been induced by the heated control. Occasionally a person will be found who reacts to heated toxins. Such reactions are discounted if none are induced in the other persons tested.

Diluent.—Borate-buffered sodium chloride solution, pH 8.4, is used as diluent (*F66*). The amount required is dispensed and sterilized in graduated Pyrex bottles.

When all the reagents are at room temperature, add sufficient sterile distilled water to the buffered diluent to restore the volume lost by evaporation during sterilization. Pipette the required amount of toxin to give a dilution containing 10 S.T.D. per milliliter, then mix thoroughly by rotating the bottle. Pass through a Mandler filter candle, discard the first 500 ml. filtered, and stop the filtration before the inner glass cylinder starts to empty. Remove samples for potency tests on animals and persons, and make the required sterility tests.

Dilute the toxin for the heated control similarly with the same lot of diluent. Before filtration, however, heat in streaming steam at one atmosphere of pressure for fifty minutes, see "Sterilization," p. 241. After the material has cooled, filter according to the procedure for the diluted toxin. Remove a sample for control tests on animals and persons and make the required sterility tests.

If the results of the tests are not satisfactory, the material is discarded.

STANDARDIZATION OF TOXIN DILUTION

Preliminary potency tests are made on animals in comparison with the standard toxin. See "Production and Standardization of Streptococcus

Toxin," p. 641. Inject 0.1 ml. of the toxin and heated-control dilutions and 1 S.T.D. of the standard toxin, heated and unheated. After the sterility and animal tests are completed, confirmatory tests are made on at least 5 susceptible persons (preferably of different degrees of susceptibility) and on 5 insusceptible persons.

PREPARATION FOR DISTRIBUTION

When the potency tests are completed, both the diluted toxin and the heated-control material are dispensed in 1.5- and 5-ml. volumes. The material if kept under proper conditions is considered satisfactory for use for one year from the date of the satisfactory potency test following dilution. The date is not extended beyond this time; any material remaining is discarded.

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CHAPTER 70

PRODUCTION AND STANDARDIZATION OF SERA AND OTHER PREPARATIONS FOR DIAGNOSTIC USE

Univalent and multivalent sera for use as diagnostic reagents or for the differentiation of groups or types of certain bacterial species are produced by the immunization of horses or rabbits against specific group or type strains. Methods for the production of these sera and for other preparations used in tests that aid in diagnosis are given in this chapter.

MISCELLANEOUS SERA

Sera are produced against the following microorganisms:

- *Brucella abortus*
- *Bacterium alkalescens*
- *Bacterium dysenteriae* (Newcastle)
- *Bacterium dysenteriae* (Shiga)
- *Bacterium dysenteriae* (Schmitz)
- *Bacterium dysenteriae* (Sonne)—rough and smooth
- *Proteus* X
- *Bacterium tularense*
- *Bacterium typhosum*
- *Neisseria meningitidis*, Groups I, II, and IIa
- *Salmonella paratyphi*-A, *Salmonella paratyphi*-B, and other species of *Salmonella*
- *Bacterium dysenteriae* (Flexner and Shiga) multivalent serum
- *Neisseria meningitidis*—multivalent serum
- Microorganisms of other species as required

Multivalent antimeningococcus serum is produced by the immunization of horses; present supplies of multivalent (Flexner and Shiga) antidysentery serum and sera for *Bact. typhosum* and *S. paratyphi*-A and *S. paratyphi*-B were also produced in horses; all other sera in rabbits.

Normal horse and rabbit sera are supplied for control purposes.

Botulinus antitoxic sera and diphtheria antitoxin are also available for diagnostic use. For their production, see chapters on botulinus antitoxic sera and diphtheria antitoxin.

Maintenance of Strains.—Representative strains of as high antigenic activity as possible are selected and maintained (see "Collection of Type Cultures," p. 87) for use in the production of the sera.

IMMUNIZATION OF RABBITS

Normal rabbits weighing in general from 1800 to 3000 grams are used, depending upon the serum to be produced. A trial bleeding is taken before immunization and the serum is held for reference. Three methods of immunization are employed: Method I requires sixteen days, Method II, twenty-eight days, and Method III, used for the production of univalent antimeningococcus serum, twenty-three days. The rapid method is usually preferred but Method II may be found more satisfactory with highly toxigenic strains, such as *Bact. dysenteriae* (Shiga) and certain strains of *Salmonella*. In all three methods preliminary injections of heated suspensions are given, followed by injections of live microorganisms. Equally satisfactory results have been obtained by substituting salt-solution suspensions of microorganisms killed with 0.25-per-cent formalin throughout the course of immunization by Methods I and II, with gradual increases in the dosage in Method II. With certain species, reduction of the dosage is sometimes necessary. If after one series of injections the serum from the trial bleeding is not of sufficient titer, an additional series is given, the amount of the injections being based on the condition of the animal. All injections are given intravenously.

- **Method I.**
    - 1st and 2d days: $\frac{1}{10}$ of a slant, killed culture
    - 5th, 7th, and 9th days: $\frac{1}{10}$ of a slant, live culture
    - 14th day: trial bleeding
    - 15th day: bleed out
- **Method II.**
    - 1st day: $\frac{1}{200}$ of a slant, killed culture
    - 4th day: $\frac{1}{100}$ of a slant, killed culture
    - 7th day: $\frac{1}{50}$ of a slant, killed culture
    - 15th day: $\frac{1}{200}$ of a slant, live culture
    - 18th day: $\frac{1}{100}$ of a slant, live culture
    - 21st day: $\frac{1}{50}$ of a slant, live culture
    - 26th day: trial bleeding
    - 27th day: bleed out

Before beginning immunization, identify and determine the purity of the culture to be used. Transfer a small loopful of growth to a tube of meat-infusion broth (*F2A*). Mix and streak one loopful of the suspension on two beef-infusion agar plates (*F33*), and incubate overnight. If the colony formation is uniformly characteristic, use the original culture for further subculture. If, however, the plate contains a mixture of so-called rough and smooth elements, fish two typical, smooth colonies to appropriate media. Test the original culture or a fishing for agglutination in homologous immune serum. From the selected culture inoculate two or more slants, *F33*, (133- by 16-mm. tubes), for the immunizing doses and for the seed for further subcultures. After from sixteen to eighteen hours' incubation, make a slide preparation, stained by Gram's method, of the transplants to be prepared to assure purity. Add 4 ml. of salt solution to each slant and scrape off the growth with a loop. Filter the suspension through a cotton filter previously moistened with 2 ml. of salt solution. Rinse the filter with 4 ml. of salt solution.

If microorganisms killed by heat or formalin are to be given, prepare sufficient material at one time for the required number of doses in the series. For heat-killed, heat the suspension at 60°C. for thirty minutes. Streak a blood-agar plate (*F43A*) and inoculate one tube of aerobic "sterility-test" broth (*F3B*) with 0.1 ml. of the heated suspension. For formalin-killed, prepare the suspension as above using salt solution containing 0.25 per cent of formalin, store in the cold room for from twenty-four to forty-eight hours and test for viability. Dilute a portion of the suspension, each milliliter of which corresponds to $\frac{1}{10}$ of the slant, so that the required dose is contained in 1 ml. (for $\frac{1}{25}$ of a slant, dilute 1 + 1.5; $\frac{1}{50}$, 1 + 4; $\frac{1}{100}$, 1 + 9; $\frac{1}{200}$, 1 + 19). Inject 1 ml.

The following modifications in Method I have been found satisfactory for the production of certain sera:

Br. abortus. A suspension of 24- to 48-hour cultures heated to 60°C. for one hour is used throughout the course of immunization. The doses are: 1st and 2d, $\frac{1}{2}$ of a slant; 3d and 4th, 1 slant; 5th, 2 slants.

Bact. tularense. Cultures are grown for forty-eight hours on cystine beef-infusion agar, *F35B*, (150- by 19-mm. tubes). The microorganisms are suspended in salt solution containing 0.25 per cent of formalin and stored in the cold room for seventy-two hours. The doses are: $\frac{1}{10}$, $\frac{1}{5}$, $\frac{1}{2}$, 1, and 1 slant.

Method III.—Univalent antimeningococcus sera, groups I, II, and IIa are produced with standard strains for use in group differentiation of meningococci and for control purposes in standardization tests. Sera against additional strains are produced as required.

1st day: $\frac{1}{100}$ of a slant, killed culture
3rd day: $\frac{1}{100}$ of a slant, live culture
5th and 12th days: $\frac{1}{50}$ of a slant, live culture
14th and 16th days: $\frac{1}{25}$ of a slant, live culture
21st day: trial bleeding
22d day: bleed out

With certain strains of meningococci, those of group II particularly, increased dosage may prove more satisfactory. For sera to be used in precipitation and capsular-swelling tests, the series of doses is usually repeated after a rest period of three or four weeks.

To prepare the cultures, inoculate a beef-infusion serum glucose agar slant, *F40A*, (150- by 19-mm. tube) and incubate this and subsequent cultures for from sixteen to eighteen hours. Make a slide preparation stained by Gram's method. For strains not in active use, carry out further identification and purity tests as described for antimeningococcus serum (horse), p. 698. If satisfactory, inoculate two serum glucose agar slants.

Use one as a seed for further transplants. Prepare the culture suspensions as for Methods I and II. If killed microorganisms are to be given, heat the suspension at 65°C. for thirty minutes.

Bleedings.—Take a trial bleeding of 4 or 5 ml. from the marginal ear vein and test the serum immediately. If the titer is adequate, bleed out the rabbit from the heart or if it is desirable to keep the animal, take a smaller volume.

IMMUNIZATION OF HORSES

For the production of multivalent antidysentery serum and sera for *Bact. typhosum* and *S. paratyphi*-A and *S. paratyphi*-B the following method was used: grow the cultures on beef-infusion agar, *F33*, (133- by 16-mm. tubes) and suspend in salt solution. The preparation of the doses is similar to that described for antimeningococcus serum, p. 699, except that 2 instead of 3 ml. of salt solution are added to each slant and the filter is moistened with a volume of salt solution equal to 1 ml. for each slant and rinsed with the same volume. Heat the suspension of microorganisms in a water bath at 60°C. for thirty minutes. Streak a blood-agar plate and inoculate one tube of aerobic sterility test broth with 0.1 ml. of the heated suspension. Injections are made intravenously. Series of 3 daily injections are given with an interval of seven days between series. The dosage is usually 1 ml. the first day, followed by slight increases in succeeding injections depending upon the temperature reactions. For the production of multivalent serum, begin immunization with one strain, preferably the less toxic, and gradually add the other strains in later series. Live cultures are substituted for killed microorganisms only if the latter fail to produce a satisfactory agglutinating serum.

For the production of multivalent antimeningococcus sera, see "Production and Standardization of Antimeningococcus Sera," p. 697.

PREPARATION OF SERA

Rabbit serum is drawn off from the clot and centrifuged to clarify. These sera may be preserved with 50 per cent of glycerol, when such dilution will not decrease the titer of the serum too much, or with other preservative, at present 1-per-cent 'Merthiolate' to give a final concentration of 0.01 per cent. Normal horse and normal rabbit sera prepared for control purposes are also preserved with 50 per cent of glycerol or other preservative. Sterility tests are made of each serum. From 1 to 2 ml. are removed for potency tests. Contaminated serum is filtered at once and tests of sterility made. Otherwise, sera are stored in the cold room until required. If necessary, filter serum to clarify before dispensing.

Horse serum is treated as described in "Care and Use of Large Animals."

p. 67, and cresol to give a final concentration of 0.3 per cent is added as described for antimeningococcus serum, p. 700. Glycerol to give a 50-per-cent concentration is also used in untreated horse serum or in that which already contains cresol.

STANDARDIZATION OF SERA

The sera are standardized by determining the agglutinative titer. A serum is tested by the macroscopic tube agglutination test against its homologous strain and if indicated against related strains to determine the limit of group agglutination. Before release, samples of all sera are tested, as far as is practicable, with recently isolated homologous strains under conditions in which they are to be employed finally. The titer, estimated conservatively, is based on the highest dilution in which agglutination occurs with the homologous strain.

Agglutination Tests.—The procedures for the macroscopic tube agglutination tests are in general the same as those described in "General Bacteriologic Technic," (see p. 19). A serum that has previously given satisfactory results is used as a control. The normal control serum is tested in a dilution equal to the lowest dilution of the test serum.

Serum produced against *Br. abortus* is standardized with alcohol-treated suspensions; that produced against *Bact. tularense*, with formalin-treated suspensions.

The period and temperature of incubation of the serum-culture mixtures vary with certain microorganisms as follows: In the case of bacillary incitants of enteric diseases, incubate the mixtures at 48°–52°C. for from fifteen to twenty hours; of *Br. abortus* at 48°–52°C. for four hours and store in the cold room overnight; of *Bact. tularense*, *Proteus*, and *N. meningitidis* at 34°–36°C. for two hours and store in the cold room overnight.

PREPARATION FOR DISTRIBUTION

Diagnostic sera are dispensed in 2-, 2.5-, and 5-ml. volumes.

PREPARATION OF SERA FOR IDENTIFICATION OF SALMONELLA

Sera prepared by the following methods have been satisfactory, but with further experience modifications will probably be incorporated. For explanation of the antigenic formulas, see p. 305.

Multivalent Salmonella Serum.—Combine equal parts of agglutinating sera produced with *Salmonella paratyphi*-A ([I].II.XII...a—), *S. paratyphi*-B ([I].IV.[V].XII...b↔1, 2...), *S. paratyphi*-C (VI.VII...c↔1, 5...), *S. enteritidis* ([I].IX.XII...g, m...—), *S. london* (III.X.XXVI. 1, v↔1, 6...), and *S. senftenberg* (I.III.XIX.g, s, t...—). Dispense in 2-ml. volumes.

[ ] These antigens may be absent
... Abbreviated formulas
↔ Diphasic, in the mixed phase both flagellar antigens are present; in phase 1, only the first, and in phase 2, only the second.

A serum prepared in this way has been found to agglutinate all of the common species of *Salmonella*. Although there is duplication of many of the antigens, none of the sera should be omitted unless this is indicated by further experience.

Sera for Identifying S. paratyphi-A and S. paratyphi-B.—Prepare "0–II" serum by absorbing *S. paratyphi*-A ([I].II.XII...a—) serum with a suspension of *S. bispebjerg* (I.IV.XII...a↔e, n, x...) mixed phase or phase 1 in salt solution containing 0.2 per cent of phenol.

Prepare "0–IV.V" serum by absorbing *S. paratyphi*-B ([I].IV.V.XII... b↔1, 2...) with suspensions of *S. paratyphi*-A (I.II.XII...a—), *S. kirkee* (XVII.b↔1, 2...) in the mixed phase aud *S. kentucky* (VIII.XX.i↔z_6) in salt solution containing 0.2 per cent of phenol.

The role of *S. kentucky* is not clear but it removes unidentified agglutinative properties for itself and other species.

Prepare "H–b" serum by absorbing *S. paratyphi*-B ([I].IV.V.XII... b↔1, 2...) phase 1 or mixed-phase serum with *S. typhi-murium* (I.IV.V. XII...i↔1, 2, 3...) phase 2 or mixed phase in salt solution containing 0.2 per cent of phenol.

Prepare "H–1, 2..." serum by absorbing *S. paratyphi*-B ([I].IV.V. XII...b↔1, 2...) phase-2 serum with (a) a suspension of *S. paratyphi*-B heated for two hours at 100°C. in an autoclave and (b) a suspension of *S. gallinarum* (IX.XII...—.—) in salt solution containing 0.2 per cent of phenol.

Absorption Technic. Prepare a 1:5 dilution of the serum in salt solution containing 0.2 per cent of phenol and combine with an equal volume of a heavy suspension of the culture grown for from twenty to forty-eight hours on beef-infusion agar (*F34A*). Incubate for from two to six hours at 35°–37°C., shaking occasionally. Store in a refrigerator for from eighteen to forty-eight hours and centrifuge. Decant the supernatant and add 0.005-per-cent 'Merthiolate'. Filter through infusorial earth in a Büchner funnel and through a final filter candle. Test for sterility and dispense in 5-ml. volumes.

ANTIPNEUMOCOCCUS SERA FOR PNEUMOCOCCUS TYPE DIFFERENTIATION

Sera for pneumococcus type differentiation are produced in rabbits and are standardized for use in the microserologic technic of Neufeld (1).

Sera are distributed for types 1 through 33 with the exception of types 26 and 30 Because some of the therapeutic sera contain antibody activity for several closely related types, the activity for these types has not been absorbed from the corresponding sera for type differentiation. The sera, therefore, react also with the related types, for example: Type 6, 6B, and No. 39301 (unclassified, related to type 6); type 7, 7A; type 9, 9L; type 11, 11A, and No. 3814, probably 11B; type 15, 15A, and 15B; type 18, 18A, and 18C; type 22, 22A; type 23, 23A; type 24, No. 39589 (unclassified, related to type 24); type 29, 35B; type 33, 68.

In addition, for special investigations, univalent sera are prepared for the types described by Cooper (2), Walter (3), Kauffmann (4), Mørch (5), and Eddy (6) for which representative strains are available.

PRODUCTION OF SERA

Rabbits are immunized and the sera prepared by the methods described for the production of therapeutic antipneumococcus rabbit serum, p. 691.

STANDARDIZATION OF SERA

All sera are tested for potency with a representative strain of the homologous type and for cross reactivity with pneumococci of the heterologous types. When cross reactivity is present, it is removed by absorption.

Maintenance of Strains.—The representative strains of the types in active use are maintained in beef-infusion semisolid agar (*F36C*) with rabbit serum. Each culture is passed through a mouse at least once a month.

Preparation of Antigens.—Antigens prepared by two methods are used; suspensions of peritoneal washings from a mouse and the formalinized cell suspensions described by Eddy (7, 8).

Mouse Peritoneal Washings: Inject a mouse intraperitoneally with a lethal dose of a 16-hour culture in infusion broth (*F5A*). From four to sixteen hours later, chloroform the mouse and wash the peritoneal cavity with salt solution in a capillary pipette fitted with a rubber bulb. Dilute the suspension of peritoneal washings with salt solution to a turbidity approximately equal to glass standard No. 1. Examine for typical capsular swelling. If the mouse dies and is autopsied within from two to four hours, the suspensions are usually satisfactory. These cells autolyze rapidly and are satisfactory for use only during the day on which they are prepared.

Formalinized Suspensions.—Inoculate a tube of infusion broth (*F5A*) from the latest serum semisolid culture or with the heart blood of a mouse injected previously with the test strain and incubate at approximately 36°C. for from fifteen to sixteen hours. Plate on blood agar (*F43A*). Examine a slide preparation stained by Gram's method and, if the culture appears to be pure, inoculate with 1 ml. of the seed culture a tube containing 14 ml. of infusion broth (*F5A* plus 0.5 per cent of glucose) previously warmed to incubator temperature. Incubate at 36°C. until the turbidity approximates that of glass standard No. 3, usually from three and one-half to five hours. Add formalin to a concentration of 1.43 per cent of the broth volume and let stand in the cold room overnight. If sedimentation occurs, decant the supernatant and discard the sedimented cells. Dilute the supernatant suspension with peptone solution (*F60.5*) to equal glass standard of turbidity No. 1. Examine for typical capsular swelling. Store in the cold room. Antigens prepared by this method have been found to be satisfactory for a period of several months.

Technic of Test: Place one loopful of antigen on a glass slide with a loop approximately 1 mm. in diameter. Using a loop from 5 to 6 mm. in diameter, mix one loopful of serum thoroughly with the antigen. Add to this mixture one drop of alkaline methylene blue. This may be conveniently added with a 1-ml. tuberculin syringe and a 26-gauge needle. Place a cover glass over the preparation and seal with vaseline. After one-half hour at room temperature, examine with an oil-immersion lens and reduced light from a strong electric lamp.

Record reactions as follows:

4+ maximum capsular swelling. This occurs with only a few types, such as 3 and 8
3+ slightly less than maximum capsular swelling
2+ clear-cut capsular swelling but narrower than 3+
+ slight but readily discernible capsular swelling
± doubtful capsular swelling. No distinct outline of capsule.

The degree of capsular swelling varies with the type and even with different strains of the same type. The density of the cell suspension used as an antigen is of great importance, especially in the detection of slight cross reactions with heterologous types. The antigen should be sufficiently dilute so that there are not more than two or three cells in each microscopic field.

Absorption.—Sera in which cross reactivity is demonstrated are made type specific by absorption with microorganisms of the cross-reacting strains.

Preparation of Suspension: Inoculate a tube of infusion broth (*F5.4*) from the most recent semisolid culture and incubate at approximately 36°C. for from fifteen to sixteen hours. From this culture, inoculate a flask of 10-per-cent infusion broth (*F5.5*) and incubate from eight to ten hours at 36°C. Plate on blood agar. Examine a slide preparation stained by Gram's method and, if the culture appears pure, inoculate a flask containing the necessary volume of 10-per-cent infusion broth (*F5.5*) previously warmed to 36°C., using 3 ml. of the seed culture for every 100 ml. of medium. Incubate for from five to six hours at 36°C. Plate on blood agar, add formalin to a concentration of approximately 1 per cent, and allow to stand at room temperature overnight. Centrifuge and resuspend the packed cells in salt solution containing 0.5 per cent of formalin. Make up to a volume approximately one-fiftieth that of the original broth culture. Dilute a measured small amount of the suspension to a density equal to glass standard No. 3 and record the dilution factor. These suspensions have been found to be satisfactory for use as long as the cells give a characteristic capsular swelling when mixed with the homologous serum.

Determination of Absorbing Dose: Prepare small amounts of cell suspensions of varying densities, for example, 1, 2, 3, 5, and 10 times glass standard No. 3. Centrifuge 0.5 ml. of each suspension and remove the supernatant. Wash the cells once with salt solution to remove the formalin. Mix 0.5 ml. of the serum to be absorbed with the packed cells from each suspension. Incubate for one-half hour at 36°C. and allow to stand in the cold room overnight. Centrifuge each mixture. Remove the supernatant and test for capsular swelling with the strain used for absorption. Choose as the absorbing dose the one that completely absorbs the cross reactivity and calculate the amount of cells needed to absorb the total volume of serum.

Procedure for Final Absorption: Centrifuge the estimated amount of the absorbing suspension and discard the supernatant. Wash the packed cells once with salt solution. Centrifuge and discard the supernatant. Add the serum to be absorbed to the packed cells, mix well, incubate for one-half hour at 36°C., and allow to stand in the cold room overnight. To check the completeness of the absorption, centrifuge a small portion of the mixture of serum and cells and test for capsular swelling with the pneumococcus strain used for absorption. A serum may be absorbed with the packed cells of several types in one procedure. When all cross reactivity for the strains used for absorption has been removed, centrifuge the mixture and filter the supernatant. After filtration, determine the titer by testing successive dilutions of the serum for specific reactivity with the homologous type in the microserologic test of Neufeld.

PREPARATION FOR DISTRIBUTION

The sera for distribution must induce a prompt and characteristic capsular swelling of pneumococci of the homologous type in a satisfactory dilution and, with the exceptions noted, be free from cross reactivity.

To facilitate type differentiation, in addition to the single sera, six pools are prepared for use in preliminary testing. The pools are planned so that sera of closely related types are included in the same combination.

Pool 1	types 1, 2, 3, 5, 8
Pool 2	types 4, 6, 7, 14
Pool 3	types 10, 10A, 11, 13, 20, 29, 31
Pool 4	types 16, 17, 18, 19, 28
Pool 5	types 9, 12, 15, 22, 25, 33
Pool 6	types 21, 23, 24, 27, 32

The pools are dispensed in 2-ml. amounts and the type sera in 1-ml. amounts. After dispensing, and before release, tests for type specificity are made on each lot of serum.

SERA FOR THE CLASSIFICATION OF STREPTOCOCCI

Antistreptococcus sera for the classification of strains of the different serologic groups and the types within group A are produced in rabbits and tested by the precipitation method with extracts of representative strains. In general, the methods are similar to those developed by Lancefield (9, 10, 18–24) as reported in publications and in personal communications.

SERA FOR CLASSIFICATION ACCORDING TO GROUP

Maintenance of Strains.—Representative strains of groups A–N (9–15) are maintained in the dry state, preferably in the glossy or rough phase. For groups in which serologic types have been differentiated, strains of at least two types are preserved to test the group-specific reactivity of the serum.

PRODUCTION OF SERA

Preparation of Cultures.—Formalin-treated streptococci suspended in salt solution, in 1-per-cent dipotassium phosphate (K_2HPO_4), or in Locke's solution (*F63*) are used for the immunization of rabbits. Antistreptococcus sera of groups D and F have been difficult to prepare. Moderately potent antisera of these groups have been produced using Locke's solution as a suspending medium for the streptococcus cells. Preliminary studies indicate that 1-per-cent K_2HPO_4 may also be satisfactory. Serum from bleedings taken before immunization is tested with extracts of representative strains of all groups. If the serum is found to react with the extract of any group, the rabbit is immunized with a strain of that group.

Use as inoculum the 4th serial transfer in beef-infusion broth (*F5A*) from a dried preparation or from a stock culture in infusion broth (*F5A* plus 2-per-cent defibrinated normal rabbit blood) inoculated within four weeks from a dried preparation. Streak a blood-agar plate (*F43A*) from the dried or stock culture when the first transfer is made to determine its freedom from contamination. Incubate the seed culture for from six to eight hours at approximately 35°C. Examine a slide preparation stained by Gram's method, and streak a blood-agar plate. If the plate inoculated from the original culture and the film from the seed culture show no evidence of contamination, inoculate the required amount of broth (*F5A'* plus 0.75-per-cent buffered glucose). A supply sufficient for four weeks is usually made. Group N cultures are incubated at 30°C. for sixteen hours, streptococcus group H for twelve hours at 35°C., all others at 35°C. for sixteen hours.

Examine a slide preparation stained by Gram's method and inoculate a blood-agar plate. Add to the broth culture formalin to give a concentration of approximately 1 per cent. Incubate for two hours at 35°C. and allow to stand at room temperature overnight. Eight hours after the addition of formalin, inoculate one aerobic tube of sterility-test broth (*F3B*) and a blood-agar plate. If there is no evidence of growth in the sterility-test broth after sixteen hours at 35°C., centrifuge the formalinized culture and resuspend the sediment in an amount of diluent equal to one-tenth of the original volume of broth culture.

If the culture is viable after eight hours' exposure to formalin, as has been noted, for example, with streptococci of group H, repeat the sterility test after twenty-four hours' exposure to formalin at room temperature. Store the formalinized culture in the cold room. When there is no evidence of growth, centrifuge and resuspend as described. Inoculate one aerobic tube of sterility-test broth and a blood-agar plate. Adjust the turbidity of the suspension to three times that of glass standard No. 6. Inoculate one aerobic tube of sterility-test broth and a blood-agar plate with the adjusted suspension and examine a stained slide preparation. If there is no evidence of growth or contamination, the vaccine may be used for the immunization of rabbits.

IMMUNIZATION OF RABBITS

Injection and Dosage.—Injections are given in the marginal ear vein on five successive days with a nine-day interval between each series of five injections. In the initial series the first three doses are 0.25 ml. of vaccine, the remainder 0.5 ml. Those of the succeeding series may be increased by 0.5 ml. in each series up to a maximum of 3.0 ml. After four or five series of injections, the rabbit may be rested for two months and then reimmunized. The first two doses given in the reimmunization series are 0.25 ml., the third and fourth, 0.5 ml., and the fifth, 1.0 ml. The second series is started with 1.0 ml. and the amount increased every second dose to a maximum of 3.0 ml.

Bleedings.—Trial bleedings of 5 ml. are taken from the ear vein of the rabbit before immunization is started and from four to five days after the last dose of each series. When precipitation tests indicate that the serum is of satisfactory potency, a bleeding of 40 ml. may be taken from the heart on the following day, or the animal may be exsanguinated.

Bleedings are taken into 500-ml. Erlenmeyer flasks by the method described for antipneumococcus rabbit serum, p. 692. After the clot has formed at room temperature, it is rimmed and placed in the cold room overnight. The serum is drawn off, centrifuged twice, and measured. 'Merthiolate' to give a final concentration of 0.01 per cent is added and the serum is stored in the cold room. The clot is left in the cold room another twenty-four hours and the serum is removed and used for immediate tests of potency and specificity.

STANDARDIZATION OF SERUM

Serum for distribution must be of adequate potency and free from cross reactivity. A serum of adequate potency is one that, with a satisfactory extract, produces a well-defined ring in ten minutes, a dense cloud, or transparent disc or shreds after two hours at 35°C. followed by eighteen hours in the cold room. The potency and specificity of serum from rabbits immunized with representative strains of streptococci of groups A–N are tested by the precipitation method, using as antigen extracts of the cells of representative strains of all groups.

Trial bleedings of rabbits under immunization are tested with the extracts of at least two strains of the homologous group of at least two serologic types, if these are available, and with an extract of a heterologous group, choosing one with which it is known that cross reactivity is most likely to occur. The serum from large bleedings is tested with extracts of strains of different types within the homologous group to determine the group reactivity and with extracts of at least one strain of each of the other groups to demonstrate cross reactivity.

Preparation of Extracts of Cells.—The seed culture is made as described under preparation of vaccine. If the plate inoculated from the original culture and the film from the seed culture show no evidence of contamination, inoculate 50 ml. of infusion-free medium (*F21C* plus thiopeptone). Incubate for sixteen hours at 35°C. Examine a slide preparation stained by Gram's method and inoculate a blood-agar plate (*F43A*). Centrifuge. Discard the supernatant. Dilute *N* hydrochloric acid 1:20 with salt solution. Suspend the cells in 10 ml. of this solution. Heat for twenty minutes in a boiling water bath, shaking the tubes frequently to keep the cells in suspension. Cool quickly against running water. Centrifuge, decant the supernatant, and add to it 0.25 ml. of 0.04 per cent bromothymol blue as indicator. Prepare color standards by adding 0.05 ml. of the indicator to 2 ml. of the buffer solutions of pH 7.0 and 7.2. By comparison with these standards, adjust the fluid to from pH 7.0 to 7.2 with *N* sodium hydroxide. Centrifuge. Before use, test the extract undiluted and diluted 1:2 and 1:4 with an antiserum of the homologous group and with normal rabbit serum. If specific reactivity is slight or absent, the extract is discarded.

PRECIPITATION TEST

Pipette 0.05 ml. of serum into tubes (100 by 8 mm.) and add an equal amount of the extract to form a layer on the serum. Incubate for ten minutes at 35°C. Record the presence and character of the ring precipitate. Shake the tubes, reincubate the test for two hours at 35°C., an read and record the amount and character of precipitation. The test is read and recorded again after eighteen hours in the cold room.

PREPARATION FOR DISTRIBUTION

The sera for classification according to group are dispensed in 2.5- or 5-ml. amounts. After filling and before release, tests for specific reactivity are made on each lot of serum.

SERA FOR CLASSIFICATION ACCORDING TO TYPE

Maintenance of Strains.—Representative strains of the various types of group-A streptococci are maintained in the dry state (p. 90). They are preferably in the mucoid or matt phase and when they are to be used for the production of vaccine, at maximum virulence (16–24).

PRODUCTION OF SERA

Preparation of Cultures.—Heat-killed streptococci suspended in salt solution are used for the immunization of rabbits. Trial bleedings taken before immunization are tested as described in "Sera for Classification According to Group," p. 793. If any group reactivity is demonstrated, the rabbit is not used.

Use as inoculum a mouse-heart-blood culture of such virulence that 10^{-4} ml. or less will kill a mouse within forty-eight hours. When the virulence of a strain cannot be increased to this degree by mouse passage, it is used for vaccine production after 30 or more serial passages. Streak a blood-agar plate (*F43A*) and examine a preparation stained by Gram's method. If there is no evidence of contamination, inoculate the required amount of beef-infusion broth (*F5A′* plus 0.2-per-cent buffered glucose). A supply sufficient for eight weeks is usually made. Incubate for twelve hours at 35°C. Streak a blood-agar plate and examine a slide preparation stained by Gram's method. If the plate inoculated from the seed and the slide preparation from the broth culture show no evidence of contamination, determine the reaction of the culture by the spot-plate method (25) using universal indicator (0.133-per-cent alcoholic), adjust to pH 7.0 with *N* sodium hydroxide, and heat in a water bath for thirty minutes at 56°C. Centrifuge, discard the supernatant, and suspend the cells in an amount of salt solution equal to one-tenth the original volume of broth culture. Filter through cotton. Inoculate one aerobic tube of sterility-test broth (*F3B*) and a blood-agar plate. If after eighteen hours there is no evidence of growth, adjust the turbidity of the suspension to three times that of glass standard No. 6.

Examine a slide preparation of the adjusted suspension stained by Gram's method and inoculate one aerobic tube of sterility-test broth and a blood-agar plate. If there is no evidence of contamination, the vaccine is considered satisfactory for use in the immunization of rabbits.

IMMUNIZATION OF RABBITS

Injection and Dosage.—Injections are given in the marginal ear vein. For the preliminary immunization, rabbits receive four series with a four-day interval between each series. A series consists of three doses given on successive days. After the preliminary immunization, bleedings are taken five days after the last dose of each series. The next series is begun from two to five days following the bleeding. The dose of vaccine in the initial series is 0.5 ml. and in the three succeeding series 1.0 ml. The doses in the later series may be increased by 0.5 ml. in each series to a maximum of 2.0 ml.

After approximately eight series of injections, the rabbit may be rested two months. Before animals are reimmunized, they are desensitized with a single intraperitoneal dose of 0.5 ml. Reimmunization is begun following a one-day rest period. Series are given at eight- or nine-day intervals. The dose in the first series is 0.5 ml. Doses in the later series may be increased by 0.5 ml. in each series to a maximum of 2.0 ml.

Bleedings.—Trial bleedings of 5 ml. are taken from the ear vein of the rabbit before immunization is begun and five days after the fourth and later series. When precipitation tests indicate that the serum is of adequate potency, a bleeding of 40 ml. may be taken from the heart on the following day.

STANDARDIZATION OF SERUM

Sera for the determination of type must be of adequate potency and free from cross reactivity.

The potency and specificity of the sera are tested by the precipitation method, using as antigens partially purified M extracts of representative strains.

Trial bleedings of the animal under immunization are tested with the homologous-type extract, with a heterologous-type extract with which the type is known to cross react frequently, and a group-A extract prepared from a strain of a heterologous type. Large bleedings are tested for potency and specificity, using a homologous-type extract, a heterologous-type extract of a strain with which serum of the type frequently reacts, and a group-A extract. Bleedings of satisfactory potency are pooled and tested for cross reactivity with extracts of representative strains of all available types.

Preparation of Extract.—Use as inoculum the fourth serial transfer in beef-infusion broth (*F5A′* plus 0.2-per-cent buffered glucose) from a dried preparation or from a stock culture in rabbit-blood broth (*F5A* plus 2.0-per-cent defibrinated normal rabbit blood) made within four weeks from a dried preparation. Mouse-heart-blood cultures have been found more satisfactory than dried preparations or blood-broth cultures for the preparation of M extracts of some types.

Streak a blood-agar plate (*F43A*) from the original culture when the first transfer is made to determine its freedom from contamination. Incubate the seed culture (50 ml.) for six hours at 35°C. If the plate from the original and a film from the seed culture, stained by Gram's method, show no evidence of contamination, inoculate 1500 ml. of 10-per-cent infusion broth (*F5.5*) and incubate for eighteen hours at 35°C. Examine a stained slide preparation and streak a blood-agar plate. Centrifuge and discard the supernatant. Dilute *N* hydrochloric acid 1:20 with salt solution. Suspend the cells in 50 ml. of the solution. Using 0.04-per-cent thymol blue as indicator, determine the pH by the spot-plate method. If necessary, adjust to pH 2.2 with *N* hydrochloric acid. Heat in a boiling water bath for ten minutes, shaking the tubes frequently to keep the cells in suspension. Check the pH. If necessary, readjust again with *N* hydrochloric acid and reheat for ten minutes. Centrifuge and decant the supernatant. Adjust with *N* sodium hydroxide by the spot-plate method to approximately pH 7.4, using 0.04-per-cent bromothymol blue as indicator. Clear by centrifugation.

Test by the precipitation method for type and group reactivity with an absorbed homologous type-specific antiserum and a group-A antiserum. If a well-defined ring is present in the precipitation test after ten minutes at 35°C., precipitate the specific protein from the extract by the following method. Dissolve from 1 to 2 per cent of sodium acetate in the extract and add an amount of 95-per-cent alcohol equal to four times the volume of dilute hydrochloric acid used. Allow to stand in the cold room for eighteen hours. Centrifuge and discard the supernatant. Partially dry the precipitate and dissolve it in 10 ml. of salt solution. Allow to stand five minutes and centrifuge. Decant the supernatant and add to it an amount of alcohol equal to three times the volume of dilute hydrochloric acid used. Allow to stand in the cold room for from one to two hours. Centrifuge, discard the supernatant, and partially dry the precipitate. Dissolve in 12 ml. of salt solution. Centrifuge. Draw off the supernatant and add to it 'Merthiolate' to give a concentration of 0.005 per cent. Test with an absorbed homologous type-specific antiserum, an absorbed heterologous type-specific antiserum, a group A antiserum, and normal rabbit serum in comparison with an extract of the same type of known reactivity.

PRECIPITATION TEST

The procedure used for testing type specificity and cross reactivity is that described under "Sera for Classification According to Group," p. 794.

To test for type-specific potency, pipette into 100- by 8-mm. tubes 0.1 ml. of undiluted serum, and add 0.2 ml. of the extract, undiluted and diluted 1:4 and 1:16, on the surface to form a layer. Record the character of the ring precipitate after ten minutes at 35 C. Shake the tubes and record the amount of precipitation after two hours at 35 C. and overnight after eighteen hours in the cold room.

ABSORPTION OF SERUM

Antistreptococcus sera for typing are absorbed with a group-A strain of a heterologous type in order to remove group and other nonspecific reactivity. If marked cross reactivity with a single heterologous type is observed, absorption with a strain of that type may be necessary (21–24).

Preparation of Absorbing Suspension.—Prepare a seed culture as in "Preparation of Extract," p. 797. Inoculate 10-per-cent infusion broth (*F5.5*) using from 2 to 3 ml. of seed culture for every 100 ml. of broth. A supply sufficient for one month may be made. Incubate for sixteen hours at 35°C. Streak a blood-agar plate and examine a slide preparation stained by Gram's method. If the plate inoculated from the seed culture and the slide preparation show no evidence of contamination, heat in a water bath at 56°C. for thirty minutes. Centrifuge and discard the supernatant. Suspend the cells in an amount of salt solution equal to approximately one-fiftieth the original volume of broth culture. Inoculate one aerobic tube of sterility-test broth and a blood-agar plate. Add sufficient 'Merthiolate' to give a final concentration of 0.005 per cent. In a 15-ml. graduated centrifuge tube determine the volume of packed cells in 5 ml. of suspension. If there is no evidence of contamination, use for the absorption of sera.

Technic of Absorption.—The satisfactory absorbing dose of the selected strain is one that completely removes reactivity with both the heterologous type-specific extracts and a crude group-A extract of a heterologous type, and that does not significantly reduce the specific reactivity of the serum.

Make three mixtures of undiluted serum and packed cells ranging from one part of serum and one part of packed cells to ten parts of serum and one part of packed cells. Use no more than 1 ml. of serum. The proportions to be used are determined by the degree of cross reactivity. Mix the serum and cells thoroughly. Incubate for thirty minutes at 35°C., shaking occasionally, and allow to stand in the cold room overnight. Centrifuge. Test the supernatant for type specificity and cross reactivity by the precipitation method in comparison with the unabsorbed serum using a homologous type extract, one or more heterologous type extracts with which the unabsorbed serum is known to react, and a crude group-A extract of a heterologous type. If the largest amount of cells used fails to remove the cross reactivity and the type-specific reactivity is strong, larger amounts of packed cells may be tried. If the cross reactivity is not removed and type specificity is reduced, cells of another strain may be tried for absorption.

Employing the strain and the proportion of packed cells found to be satisfactory, absorb an amount of serum sufficient for six month's use. The absorbed preparation is tested for type specificity and absence of cross reactivity by the precipitation method, using extracts of all available types.

The sera so absorbed may be used in the precipitation test with crude or purified extracts. They may also be used in the slide-agglutination test with cell suspensions, for example, to differentiate streptococcus strains of group A, types 10 and 12, which have a common M substance. Agglutinating sera by which these two types may be distinguished are prepared by reciprocal absorption of their sera (24).

BACTERIAL SUSPENSIONS

BACT. TYPHOSUM—B—ALCOHOL- AND FORMALIN-TREATED SUSPENSIONS

Alcohol- and formalin-treated suspensions of *Bact. typhosum* are prepared for use in demonstrating granular and floccular agglutination in tests that aid in the diagnosis of typhoid fever. Entirely smooth cultures are essential for both types of suspension. Nonflagellated strains may be found more satisfactory for the alcohol-treated suspension, while actively motile strains should be employed for those treated with formalin.

Maintenance of Strains.—The standard strains are maintained in a dry state. The motility and agglutinability of a culture can usually be enhanced by cultivation at room temperature for a few days.

Special precautions are observed when cultures of live typhoid bacilli are handled. See "General Bacteriologic Technic," p. 4.

ALCOHOL-TREATED SUSPENSION

The growth from 18- to 22-hour cultures on 2-per-cent sugar-free beef-infusion agar (*F34A′*), contained in 1-liter Roux flasks, is suspended in salt solution containing 0.5 per cent of phenol. The suspension is treated with alcohol and adjusted to a standard turbidity.

Preliminary Transfers. Identification and Purity Tests.—From the selected culture, inoculate a beef-infusion agar slant (*F33*) and a tube of beef-extract lactose-glucose agar (*F50B*), and transfer a small loopful of growth to a tube of meat-infusion broth (*F2A*). Streak a loopful of the broth suspension on two sugar-free beef-infusion agar plates. Incubate all these and subsequent cultures at from 35° to 37°C. for from sixteen to twenty-two hours. If the colony formation appears uniformly smooth and the reaction in the carbohydrate medium is typical, make from the growth on the agar slant a slide preparation stained by Gram's method. Using preferably a serum known to contain granular agglutinating properties, test a salt-solution suspension of several loopfuls of growth from the agar slant, equal in turbidity to glass standard No. 2.5 in two or more of the highest-fold dilutions that gave marked agglutination with a suspension previously found satisfactory. Incubate at 48°–52°C. for eighteen hours. If all reactions are typical, use the remainder of the agar-slant culture for further transfers. If the growth on the plates is not uniform, select three typical, smooth colonies. Inoculate from each an agar slant and a tube of lactose-glucose medium. Then make a suspension in broth of the remainder of the growth and streak an agar plate. The following day select the set of subcultures of a fishing that has shown uniformly smooth colony formation on the beef-infusion agar plates and typical reaction on the lactose-glucose medium. Test for agglutinative properties.

From the transfer on beef-infusion agar selected, inoculate one agar slant (133- by 16-mm. tube) for every two 1-liter flasks of medium to be inoculated for seed cultures and incubate. The growth from one of these is sufficient for the inoculation of 20 flasks of medium for harvesting. Pipette 5 ml. of salt solution to each slant, then transfer 2 ml. of suspension to each flask. Cover the entire surface of the medium by tilting the flask back and forth. After incubation add to each flask about 40 ml. of salt solution, then make a slide preparation stained by Gram's method. Discard any flasks that are contaminated.

Inoculation of Medium for Harvest.—Draw off any excess "water of condensation" from the flasks. With a pipette inoculate the medium in each with about 2 ml. of suspension from the seed flasks, and incubate.

Harvesting of Growth.—Add to each flask 20 ml. of salt solution containing 0.5 per cent of phenol (*F68*), and make a slide preparation stained by Gram's method. Discard any flasks that are contaminated. Pool the suspensions in a sterile graduated cylinder, then, in order to facilitate mixing, pour the measured material into a flask. To the suspension slowly add alcohol to give a concentration of $33\frac{1}{3}$ per cent and mix by rotating the flask. Volume 95-per-cent alcohol required = Volume of suspension $\times 0.54$, a constant obtained from the formula: $95X = 33\frac{1}{3}$ (volume suspension $+ X$), in which X = volume 95-per-cent alcohol required. Return the alcohol-treated suspension to the graduated cylinder and incubate overnight at from 35° to 37°C. The following morning decant the supernatant from the sediment into a bottle, inoculate 20-ml. of semisolid meat-infusion agar (*F32B*) with 0.1 ml., and streak a blood-agar plate as a test of sterility. Store in the cold room until adjusted.

Adjustment of Concentrated Suspension.—Adjust the suspension to a turbidity ten times that of glass standard No. 2.5 and to an alcohol content of 30 per cent. Make the necessary dilution as soon as sterility is assured. With salt solution dilute 0.5 or 1.0 ml. of the suspension to the density of glass standard No. 2.5. Compare the turbidity of the dilution with that of a sample of the preceding lot as finally diluted. Calculate the required volume of diluents. To the suspension add the phenolized salt solution and alcohol, and mix thoroughly. Remove a 10-ml. sample for tests.

Standardization of Adjusted Suspension.—The agglutination reactions of the freshly prepared suspension are compared in a macroscopic tube test with those of the last satisfactory lot. Dilute a sample of each 1:10 with salt solution and test them with the homologous agglutinating serum and if possible with at least six patients' sera, in three of which varying degrees of agglutination have been shown, in three none. Incubate the mixtures at from 48° to 52°C. for eighteen hours.

FORMALIN-TREATED SUSPENSION

In the preparation of the formalin-treated suspension, the growth in the flasks is suspended in buffered, pH 8.4, salt solution (*F68.5*) containing 2 per cent of formalin. The procedures are similar to those for the alcohol-treated material with the following exceptions. Examine a 16- to 18-hour broth culture for motility in a hanging drop preparation and test the salt-solution suspension preferably with a serum that gives marked floccular agglutination. To each flask of culture for harvesting add 25 ml. of the formalinized buffered salt solution. Store the pooled suspension in the cold room, decant after forty-eight hours, and test for sterility. Should growth develop, make retests.

Adjustment of Concentrated Suspension.—With the *Bact. typhosum* formalin-treated suspension, calculate the required volume of the formalinized buffered salt solution to be added to bring the suspension to a turbidity ten times that of glass standard No. 2.5.

PREPARATION FOR DISTRIBUTION

The concentrated alcohol- and formalin-treated suspensions of *Bact. typhosum* are dispensed in 10-ml. volumes (dilute 1:10 for use).

PROTEUS X—ALCOHOL-TREATED SUSPENSION

Alcohol-treated suspensions of *Proteus* X are prepared for use in tests that aid in the diagnosis of Rocky Mountain spotted fever, typhus fever, and other rickettsia infections. An entirely smooth, preferably nonflagellated culture is used. The procedures for the maintenance of *Proteus* X and for the preparation and standardization of the suspension are similar to those for *Bact. typhosum* alcohol-treated suspension except that 2-per-cent beef-infusion agar (*F34A*) is used and in the macroscopic tube test the mixtures are incubated at 35°–37°C. for two hours and stored in the cold room overnight.

BRUCELLA ABORTUS—ALCOHOL-TREATED SUSPENSION

An alcohol-treated suspension of *Brucella abortus* is prepared for use in agglutination tests that aid in the diagnosis of undulant fever. A strain selected on the basis of its agglutination reactions with sera from patients having undulant fever is used. It is maintained in the dry state. Unless otherwise indicated the procedures for preparing the suspension are similar to those for the preparation of *Bact. typhosum* alcohol-treated suspension.

Because of the highly infectious nature of the microorganism, rigid precautions should be observed throughout. See "General Bacteriologic Technic," p. 4. When practicable, the work is assigned to persons who have had a *Br. abortus* or *Br. melitensis* infection.

Preliminary Transfers and Purity Tests. From the selected culture inoculate a beef-infusion agar slant and incubate it for twenty-four or forty-eight hours depending upon the growth. Make a slide preparation stained by Gram's method. From the culture inoculate one slant (133- by 16-mm. tube) for each 1-liter flask of 2-per-cent beef-infusion agar (*F34A*) to be inoculated for seed cultures. Growth from one of these flasks is sufficient for the inoculation of ten flasks of medium for harvesting. Incubate these and subsequent cultures for forty-eight hours. Add 3 ml. of salt solution to each slant and with a pipette inoculate a flask of medium with the suspension from each. After incubation add 20 ml. of salt solution to each seed flask and make a slide preparation stained by Gram's method.

Inoculation of Medium for Harvest. With a pipette inoculate the medium in each flask with 2 ml. of suspension from the seed flasks, and incubate.

Harvesting of Growth. The procedures are the same as those for suspensions of *Bact. typhosum* except that approximately 10 ml. of salt solution containing 0.5 per cent of phenol are added to each bottle.

STANDARDIZATION

The procedures are the same as those for suspensions of *Bact. typhosum* except that the mixtures are incubated at 48°–52°C. for four hours, stored in the cold room, and readings made the following morning.

BACT. TULARENSE—FORMALIN-TREATED SUSPENSION

A formalin-treated suspension of *Bact. tularense* is prepared for use in agglutination tests for evidence of tularemia. The growth from 48-hour cultures on cystine beef-infusion agar (*F35B*) is suspended in formalinized buffered salt solution. The suspension is finally adjusted to a standard turbidity. For maintenance of strains used in its preparation, see "Collection of Type Cultures," p. 90.

Because of the highly infectious nature of the microorganism, rigid precautions should be observed throughout. See "General Bacteriologic Technic," p. 4.

Preliminary Transfers and Purity Tests

From the selected culture inoculate three cystine beef-infusion agar slants, *F35B*, (175- by 25-mm. tubes) and make a slide preparation stained by Gram's method. Incubate these and subsequent cultures for forty-eight hours. If after twenty-four hours the growth is scant and not uniformly distributed, spread it over the surface of the medium. The growth from one tube should be enough for the inoculation of 7 or 8 additional tubes of medium. Make serial subcultures until there are sufficient seed tubes for the inoculation of the material for harvesting.

Harvesting of Growth

To each tube add 10 ml. of buffered salt solution containing 2 per cent of formalin and place in a slanting position so that the entire surface of the medium is covered. After two hours, pool in a 50-ml. centrifuge tube the suspensions from every 4 or 5 slants. From each pool make a slide preparation stained by Gram's method. Store the tubes in the cold room. After twenty-four hours, spread a generous loopful of the suspension from each tube on blood-agar plates as a test for sterility. If no growth appears within forty-eight hours, centrifuge the suspensions. Resuspend the sediment in each tube in 5 or 6 ml. of the formalinized salt solution and filter through a thin layer of moistened cotton to remove particles of medium.

Adjustment of Concentrated Suspension

Calculate the required volume of the formalinized buffered salt solution to be added to bring the suspension to a turbidity ten times that of glass standard No. 2.5. The procedures are similar to those for the adjustment of *Bact. typhosum* formalin-treated suspension. Remove a 5-ml. sample for tests.

STANDARDIZATION

The suspension is standardized according to the procedure used for *Bact. typhosum* suspensions except that in the macroscopic tube test the mixtures are incubated at 35°–37°C. for two hours and stored in the cold room overnight.

PREPARATION FOR DISTRIBUTION

Bact. tularense formalin-treated suspension is dispensed in 5-ml. volumes.

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CHAPTER 71

FILTRATION OF PROPHYLACTIC, THERAPEUTIC, AND OTHER PREPARATIONS

Bacterial toxins, sera, and other preparations are routinely filtered through a candle of diatomaceous earth to remove any microorganisms present. Preliminary clarification is necessary for many products. Material such as enzyme preparations, pneumococcus culture filtrates and other antigens are concentrated and purified by ultrafiltration through nitrocellulose membranes.

Complete physical separation of antitoxins, sera, etc., and of bacterial toxins is maintained. All apparatus connected with their filtration is also kept separate. Products such as serum for human use are filtered by specially trained workers in a room designed and equipped for the purpose; bacterial toxins by the staff preparing them, in separate rooms designed for rigidly aseptic technic. For special precautions to be observed in handling infectious materials, see "General Bacteriologic Technic," p. 4.

Treatment of Material

When the material to be filtered contains precipitate, cells, or other matter that would quickly clog the pores of a filter candle, it is clarified by passage through cotton, infusorial earth or paper pulp in a Büchner funnel, or by centrifugation.

Viscous products and those containing a heavy precipitate have been found to filter more satisfactorily through infusorial earth. Some sera that contain little or no precipitate are rendered sufficiently clear by passage through cotton. Material is drawn through the filter candles by vacuum or forced through by positive air pressure. The latter is used principally in filtering sera and antitoxins, the former when less viscous fluids, such as broth cultures, or small lots of material are to be filtered. Seitz filters may be used either for clarification or final filtration of various preparations. When no preservative is present, preliminary and final filtration should be completed on the same day. Different lots of the same product may be filtered in succession through the same filter, that with the highest titer first. Treatment of the various materials is in general as follows.

Cultures (Toxins).—Broth cultures from which filtrates are prepared are usually clarified before passage through a filter candle by filtration through infusorial earth or paper pulp, asbestos wool, or filter paper and cotton. Broth cultures are then passed through a final filter candle, although occasionally it may first be necessary to further clarify them by passage through a preliminary filter, particularly tetanus and botulinus broth cultures. Vacuum is used in filtering these products. Preliminary clarification is usually unnecessary in the filtration of small amounts. The precautions required in handling infectious material are observed throughout, since the sterilizing action of the preservative is not immediate. Moreover, a filter candle may prove to be defective so that culture filtrates should be treated as infectious until the tests of sterility are completed.

Sera.—Sera to which cresol has been added are usually stored for several weeks so that precipitation may take place before filtration. All sera that contain more than a slight precipitate are passed through infusorial earth. Unconcentrated sera and concentrated "antibacterial" sera are then passed directly through the final filter candle. Should difficulty be experienced in obtaining sterile filtrates, the serum may be passed through two final filters in succession. With serum in which an unusual amount of precipitate has settled to the bottom of the bottle, it may be desirable to filter the last portion through cotton before filtration through infusorial earth. Ascitic fluid is treated similarly.

Antitoxins (Concentrated).—Antitoxins are usually stored for three months or more before preliminary filtration to permit precipitation. They are then filtered through infusorial earth and, if necessary, a preliminary filter candle before final filtration. Pressure is used for filtering antitoxin except in the case of very small volumes.

Supplies and Equipment. Paper pulp: Prepared from soft filter paper as follows: Tear 90 grams of paper (Eaton and Dykeman Co. No. 615) into small pieces and cover with distilled water. Macerate evenly but not too finely with the hands. Shake thoroughly. Pour off the water, transfer the pulp to an 8-liter bottle, and add 5 liters of 0.5-per-cent phenol solution (4500 ml. of water and 500 ml. of 5-per-cent phenol solution). If the pulp is to be used to filter toxin for the preparation of toxoid, do not add phenol. Place pulp in smaller bottles and sterilize in the autoclave.

Infusorial earth: A special high-grade earth.

Asbestos wool: Fiber, acid washed, for filtering.

Filter candles: Mandler filters (10 by 2 inches) for filtration of large volumes, small candles of standard sizes (8 by 1 inch, 5 by 1, $2\frac{1}{2}$ by $\frac{5}{8}$), Mandler and Berkefeld, for smaller volumes. Filters are graded according to permeability as follows: Mandler, preliminary filters to withstand air pressure of from 3 to 5 pounds; medium, from 6 to 9 pounds; and final, from 10 to 16 pounds. Berkefeld, preliminary filters grade V (very porous); grade N (medium); and grade W (very slightly porous).

Glass mantles: Sizes correspond to filter candles.

Apparatus for pressure filtration: As described under "Filtration by Pressure."

Cleaning solutions: See "Cleaning of Filter Candles."

Preparation for Filtration

Sterilization of Apparatus.—Apparatus used in the preliminary clarification of products must be clean but not necessarily sterile. The preliminary candles with connections and all bottles are sterilized, however.

All equipment which comes in contact with the final filtrate must be sterile It is sterilized by steam at 121° C. (2 atmospheres, 15 pounds gauge reading for thirty minutes, generally on the day preceding filtration. The candles and connections are placed in covered, copper boxes with a protecting cotton pad on the bottom. During sterilization the cover of the box is raised slightly on one side. It is closed when the autoclave is opened

Sterilization of Gowns and Caps. Care of Filtration Rooms.—See "General Instructions," p. 617.

Preliminary Filtration

Through Infusorial Earth.—Set up one or more Büchner funnels in suction flasks and connect the side arms of the flask with the vacuum system. Place in the funnel two layers of coarse, soft filter paper, such as American standard low ash No. 5160, cut slightly smaller than the bottom of the funnel but covering the holes. Dispense the infusorial earth in 7- by 1-inch glass tubes, pack it down well and plug the tubes with cotton. Dry sterilize for one hour at between 160° and 175° C. Mix the earth with sufficient physiologic salt solution or distilled water to form a thin slurry, apply vacuum, and pour the mixture into the funnel. In general the thinnest layer that is effective, for most products 15 to 20 grams, should be used for an 8-inch funnel. When a well-packed layer free from cracks is formed, place a small piece of filter paper on top and pour in the material to be filtered, letting the stream strike the paper guard. Do not allow the funnel to become empty until filtration is completed. In the filtration of bacterial toxins form the filter bed by mixing the earth with distilled water and rinse it with a small amount of the toxin. Discard the rinsings. When it is desirable to avoid dilution or loss of material, mix the earth with the material itself and refilter the first portion of filtrate. When a filter begins to clog, scraping the film from the surface of the earth may help. If not, prepare another filter.

Recovery of Antitoxins and Concentrated Sera. "Rinsings." Rinse with salt solution all bottles and suction flasks that have contained concentrated material. Combine the rinsings in a sterile bottle and attach a tag marked, "Rinsings," on which are recorded the kind of material and lot numbers. Add preservative, at present 'Merthiolate' to give a concentration of 1:10,000, and store in the cold room.

Through Pulp.—Place in the funnel a piece of hard filter paper (Whatman No. 50). To about 500 ml. of pulp (for an 8-inch funnel), add an equal volume of water and after shaking thoroughly pour the mixture into the funnel, turn on the vacuum, and place a small piece of hard filter paper on the surface of the pulp. Allow practically all of the water to drain from the pulp. A well-packed layer from 3 to 4 mm. thick is necessary to secure the best filtration. Disconnect the vacuum, and after emptying the flask rinse it with distilled water and drain thoroughly. Replace the filter, turn on the vacuum, and pour in the material to be filtered. Do not allow the funnel to become empty until filtration is completed.

In the case of toxin, free the pulp from water by passing through a small amount of the toxin, taking care to turn off the vacuum before a film forms over the surface of the pulp. Transfer the funnel to a clean flask, and place the latter in a metal container. With large lots of toxin, siphon the material into the filter, controlling the flow with a pinchcock attached to the rubber tubing of the siphon. When not in use, keep the tip of the siphon in a tube. Discard the first portion of the filtrate if it appears to be diluted with water; refilter it if cloudy.

Through Asbestos Wool.—For certain experimental work asbestos wool has been used for the preliminary filtration of broth cultures. Suspend the wool in water and proceed as in filtering through paper pulp.

Through Cotton.—Place a small disk of coarse wire in a ribbed-glass funnel; line the funnel with two thin layers of nonabsorbent cotton so arranged that the fibers cross. Pour in carefully the material to be filtered. Refilter the first portion to pass through.

Through Soft Filter Paper and Cotton.—Place a piece of soft filter paper (Eaton and Dykeman No. 615) in a glass funnel and line the paper with two thin layers of nonabsorbent cotton so arranged that the fibers cross. Pour in the material to be filtered. Refilter the first portion if not clear.

Through a Preliminary Filter Candle.—Prepare and set up the apparatus as described in the following sections for final filtration by vacuum or pressure. The rubber stopper and connections may, however, be sterilized by boiling in 0.5-per-cent cresol for from ten to fifteen minutes before the filter is set up.

Final Filtration by Vacuum

Preparation of Apparatus.—A filter candle, a glass thimble, a mantle, bottles for the filtrate, and a 2-holed rubber stopper with connections are required. The stopper is prepared by passing through one hole a straight glass tube to which is attached a short piece of heavy rubber tubing to connect with the nipple of the candle; through the second, a right-angle glass tube with a cotton guard to connect with the vacuum tubing. The outlet tube may be conveniently made from a discarded bulb pipette.

As an added precaution, 2 wide-mouthed bottles about one-quarter filled with 5-per-cent phenol solution may be placed between the cotton guard and the vacuum intake. Each bottle is fitted with a 2-holed rubber stopper. A right-angle glass tube that will extend nearly to the bottom of the bottle is passed through one hole, a shorter right-angle tube that will extend just below the stopper, through the second. The two longer tubes are connected by a short length of rubber tubing. One of the shorter tubes is connected with the outlet tube containing the cotton guard and the other with the vacuum intake.

Make certain that the cotton guard in the outlet tube is satisfactory. Boil the stopper and straight connection in 0.5-per-cent cresol. Assemble the parts so that the opening of the straight inlet tube extends below that of the outlet tube. Wire the rubber tubing on the inlet tube. Protect the stopper and the end of the rubber tubing with cotton and cheesecloth guards and wrap tin foil around the nipple end of the filter candle. Place in a copper box for sterilization.

When a small candle is to be used, the filter, mantle, and stopper are usually assembled and sterilized in a metal basket covered with paper. The lock nut, however, must not be screwed tight until after sterilization.

Procedure.—Slip a rubber washer over the nipple of the sterile candle and pass the nipple through the glass mantle. Put a second rubber washer, a metal washer, and a lock nut over the nipple and screw firmly into place. With aseptic precautions, remove the tin foil from the nipple and attach the rubber tubing that connects with the stopper; wire the connection if necessary. Support the filter and mantle on a ring stand. Insert the stopper in a sterile bottle, then wrap a strip of cotton dampened in 1-per-cent cresol over the stopper. Invert the glass thimble, slightly larger in diameter than the candle, over the candle to aid in keeping it covered with liquid. After connecting the vacuum intake to the outlet tube of the bottle or of the phenol guard bottle, turn on the vacuum gradually, and pour into the mantle the material to be filtered. Keep the candle well covered. When the bottle is full, transfer the stopper to a second bottle, at the same time closing the first with a rubber stopper previously boiled in 0.5-per-cent cresol and dried in the flame. These steps must be done rapidly and with aseptic precautions.

An alternative procedure is sometimes used when a relatively large volume of material is to be filtered. Prepare a 2-holed rubber stopper as in the previous method, using a longer piece of rubber tubing between the straight glass tube and the nipple of the candle. Omit the glass mantle. Wrap the stopper in a cotton and cheesecloth guard and place the assembled filter in a copper box or a metal basket covered with paper for sterilization. To use the filter, insert the rubber stopper, with aseptic precautions, in a sterile bottle and make connections with the vacuum line. Pour the material to be filtered into a suitable container and immerse the filter in the liquid. Turn on the vacuum gradually.

Final Filtration by Pressure

Preparation of Apparatus.—The following apparatus is required: Pressure tank with connections, filter candle and metal cylinder, sterile bottles;

also a stopper and rubber connections as described in "Final Filtration by Vacuum," except that the stopper is connected with the filter by a piece of soft rubber tubing from 2 to 3 feet long and that instead of using a cotton guard an air-outlet tube is connected with a trap consisting of a two-neck Woulff bottle three-quarters filled with 5-per-cent phenol.

The pressure tank consists of a cast-iron bowl lined with enamel and equipped with air-pressure and exhaust valves. Besides the control valve, the air-inlet line is equipped with a pressure regulating valve and a pressure gauge. The top of the tank is closed with an airtight cover held down by wing nuts. The bottom of the tank has an outlet with which the detachable metal cylinder that holds the filter candle may be connected. A stopcock controls the flow of the material into the cylinder. The bottom of the cylinder through which the nipple of the candle passes is detachable. All joints are protected by rubber washers. Three sizes of tanks are used, 4-, 8-, and 12-liter.

Boil and assemble the stopper and connections as in "Final Filtration by Vacuum." Pass the nipple of the candle through the rubber washer, then through the detached bottom of the cylinder, and finally through a metal washer and lock nut. Screw the lock nut loosely in place. Attach the rubber tubing that connects the nipple with the stopper and wire the connections. Protect the stopper and the end of the outlet tube with a cotton and cheesecloth guard. Place the filter in a copper box for sterilization.

Procedure.—Tighten the lock nut on the sterile candle, screw the base into the cylinder, and attach the latter to the tank. When using a candle smaller than 10 by 2 inches, invert over it a glass thimble before screwing it into the metal cylinder. With aseptic precautions, connect the sterile bottle and tighten all connections. Wrap the stopper in the bottle with a strip of cotton dampened in 1-per-cent cresol. Place a clean pan under the cylinder to catch possible drippings which may be returned later to the tank for filtration. Pour the material into the tank keeping the liquid-level below the air inlet. Make sure that the exhaust valve is closed and the stopcock into the cylinder open. Set the cover of the tank in position and tighten the wing nuts. With the reducing valve, turn on the pressure gradually. Do not exceed the prescribed pressure—for unconcentrated or concentrated antibacterial sera, under 15 pounds per square inch if possible, never more than 30; for antitoxins, not more than 50 pounds. Should the first portion of the filtrate appear diluted with water, add it to the residues or washings, or discard; should it contain tiny particles from the candle, refilter. Be on the alert to turn off the air pressure and to open the exhaust valve to prevent forcing air into the bottle when the tank becomes empty. When a bottle is full, shut off the pressure, open the exhaust valve, and allow equilibrium to become established.

Avoid releasing the pressure suddenly. Replace the full bottle by an empty one and stopper the former as in "Final Filtration by Vacuum."

When the material runs very slowly, indicating that the filter is clogged, shut off the pressure, open the exhaust valve, and close the valve between the tank and the cylinder. Unscrew the cylinder and pour the liquid into the drip pan. Unscrew the metal base and draw out the filter carefully. Do not detach the rubber-tubing connections. Hold the candle over a pail containing about one liter of salt solution. Brush the candle thoroughly and gently with a medium soft brush wet with the salt solution. In the case of concentrated antitoxins or sera, save the salt solution and pool with the rinsings. Hold the filter under the tap and brush it in a stream of lukewarm water, finally rinse it with distilled water. Omit this step with concentrated antibacterial sera. Replace the filter in the cylinder and after screwing the latter into position continue filtration. Return the material in the drip pan to the tank when it is next refilled. This procedure may have to be repeated, but it is preferable to substitute a second filter for the first rather than to wash frequently.

When filtration is completed, unscrew the cylinder from the tank and pour the residue into a sterile bottle.

When concentrated antitoxins have been filtered, rinse all bottles and apparatus with which they have been in contact with salt solution and pool the rinsings. In the case of concentrated antibacterial sera add the rinsings to the next lot of similar material to be concentrated. Brush the candle in salt solution. Rinse the tank with about one liter of salt solution. Force the solution through the candle and add it to the rinsings.

Concentrated serum residues and rinsings are placed in the cold room and held for reconcentration. Unconcentrated serum residues are placed in the cold room, and if considered by the worker in charge of production to be in satisfactory condition and of suitable titer, are added to the next lot of the same kind of serum filtered.

Filtration through Seitz Type Filters

There are so many modifications of the Seitz type of filter that detailed directions for its use are not given. In general, the connections to the filter and the filtration procedure are the same as for the Mandler filter.

Sterility Tests

Make routine sterility tests of all filtrates after passage through the final filters.

Care of Contaminated Apparatus and Materials

All apparatus and materials, including filter rinsings, that come in contact with pathogenic microorganisms during the process of filtration must be handled according to the procedure outlined in "General Bacteriologic Technic," p. 4. Before autoclaving, draw water through the candle and loosen the lock nut on the filter to prevent the mantle's breaking by expansion.

Sterilization of the filters by heat may be omitted when toxins of certain nonsporeforming microorganisms, such as *Corynebacterium diphtheriae,* are filtered. Wipe off the edge and outside of the mantle with one-per-cent cresol and proceed at once to clean the filter by the permanganate method.

Refiltration

Refiltration is required when the sterility tests indicate the presence of microorganisms in the filtrate or when excessive precipitation occurs on standing. Contaminated material is passed through a final filter only. The preliminary treatment of cloudy material depends upon the degree of precipitation.

Cleaning of Filter Candles

The candles may be cleaned by either of two methods. Candles used for the filtration of antitoxins and sera are cleaned by the first method, those for cultures usually by the second.

First Method.—1. Wash by brushing gently the surface of the candle with a moderately soft brush and lukewarm salt solution. If little or no film has formed on the candle, this step may be omitted.

2. Pass 3-per-cent salt solution through the candle (about 4 liters for 10- by 2-inch filter) unless salt solution has already been used.

3. Place the filter in the metal cylinder, screw the latter on the faucet, and force water through for at least twenty minutes. Brush gently. Omit this step in the case of small filters or those used for concentrated antibacterial sera.

4. Boil the candle in 2-per-cent washing soda for thirty minutes.

5. Boil in tap water for thirty minutes, changing the water several times. Use hot water when making these changes.

6. Allow the candle to cool, then hold it under the faucet and brush gently.

7. Repeat step 3 or for smaller candles set up a vacuum apparatus and draw through water.

8. Dry thoroughly. See "Drying of Candles."

Second Method—Potassium Permanganate.—Solutions for cleaning: Potassium permanganate (0.5 per cent); sodium sulfite (5 per cent); and hydrochloric acid (5 per cent). The permanganate solution may be used until the color changes to a brownish tint but it should be refiltered each time shortly before use. The sulfite solution may be used 2 or 3 times. In each step the top of the filter should be covered with the cleaning solution.

1. If the candle has been sterilized by heat, scrub it gently with a brush in tap water. If not, omit the brushing until after chemical treatment.

2. Connect the filter with a bottle. Fill the mantle with permanganate solution and apply vacuum until the filtrate becomes colored.

3. Replace the bottle by a fresh one and draw through the remainder of the permanganate solution. Substitute another bottle.

4. Pour over the candle a volume of hydrochloric acid solution mixed with an equal amount of sulfite solution. For a 10- by 2-inch candle, use 150 ml. of each. When sulfite solution is used a second or third time, the relative volume of acid should be somewhat less.

5. Place a closed cylinder over the candle and turn on the vacuum.

6. Add sufficient sulfite solution to fill the mantle, then draw it through.

7. Should brown areas remain in the candle, repeat the treatment with sulfite solution.

8. Pass tap water (at least 6 liters for a 10- by 2-inch filter) through the candle until the filtrate is free from acid as shown by litmus paper. Should tap water leave a brownish residue, scrub the candle gently with a brush.

9. Fill the mantle with distilled water and draw it through.

10. Dry thoroughly.

Drying of Candles.—Clamp the clean candle in a ring stand and connect the nipple with the vacuum inlet. Use a guard bottle. Turn on the vacuum for about twenty minutes. Dry thoroughly over a radiator or in a drying oven or incubator. If the candle is set up in a mantle, the air may be drawn through before taking the filter apart. Make sure the candles are perfectly dry. Store in wire baskets or on racks that permit free circulation of air.

Testing of New Candles

All new candles are cleaned before use. Large filter candles (10 by 2 inches) are tested when received to confirm the rated air pressure; large final filters, to determine also their sterilizing efficiency.

Cleaning.—After examining each filter carefully for obvious defects, immerse the entire candle, except the end of the nipple, in tap water and soak for from eight to eighteen hours. Remove extraneous particles by forcing water from the outside through the candle placed in a metal cylinder screwed on the faucet.

Air-Pressure Test.—When all air has been removed from the candle by soaking and rinsing, connect the filter by rubber tubing with a positive air-pressure outlet fitted with a gauge. Submerge the entire filter, nipple downward, in a glass container filled with clear water and apply air pressure gradually. Note the pressure at which air bubbles escape and observe whether they are given off evenly over the surface of the filter or at a particular point. Reject any filter that shows imperfections or does not meet the rated pressure.

Filtration Test.—To assure further the efficiency of the candle, pass through it from 3 to 4 liters of the mixture used for coagulated serum medium (Loeffler) or of serum to be used in the preparation of media, and test the sterility of the filtrate.

A suspension of microorganisms may also be used to determine the efficiency of filter candles. The filter is assembled in the usual way and a diluted broth culture of *Bacterium pyocyaneum* is filtered.

ULTRAFILTRATION

Three types of ultrafilter are used: the Seitz No. 6 for amounts of 100 ml. or less; the alumina thimble for volumes of from a few hundred milliliters to 4 liters; and the Seitz Sheet Filter, No. 20, for larger amounts. The Seitz filters are designed for use with either positive or negative air pressure. For routine work, a positive pressure of 760 mm. of mercury is satisfactory; it is maintained constant by means of a reducing valve. The alumina thimble apparatus utilizes negative pressure, usually from 60 to 100 mm. of mercury.

Nitrocellulose Membranes

The membranes used in the three ultrafilters are prepared from nitrocellulose (Parlodion) dispersed in glacial acetic acid. Definite percentages by weight—usually from 4.3 to 10 per cent—of the nitrocellulose solution are made. The time required to effect complete dispersion of the nitrocellulose in glacial acetic acid at room temperature varies from two to four weeks. The percentage composition and absolute viscosity of the solutions are carefully standardized. The percentage of nitrocellulose is controlled as follows: about 1 gram of the solution is weighed in a covered weighing bottle (5 cm. by 3 cm. high) and evaporated to dryness on a water bath; 5 ml. of redistilled acetone are added and the solution again evaporated to dryness. This process is repeated until the odor of acetic acid is no longer detectable. The residue is dried to a constant weight in a vacuum drying oven at 65°C., usually for about two hours.

The thickness of the membrane is partially dependent upon the viscosity of the nitrocellulose solutions. A solution of as low a viscosity as practicable is used so that the pore length will not be needlessly extended and the membrane thus subject to adsorption and blocking of the pores during filtration.

The viscosity of the nitrocellulose solutions is determined by the falling-sphere method with the wall correction developed by Faxén (1, 2). The apparatus in this laboratory was constructed according to the specifications of Gibson and Jacobs (3), using a discarded Carius tube and stainless-steel ball bearings. It was calibrated by means of a standardized castor oil.

The following is a typical example of the determination of the absolute viscosity of a freshly prepared 4.5-per-cent solution of nitrocellulose in glacial acetic acid, the determination being made at room temperature using a 0.1588-cm. ($\frac{1}{16}$-inch) sphere:

μ = viscosity in poises—?
S = distance of fall—15 cm.
d = diameter of sphere—0.1588 cm.
D = diameter of tube—1.95 cm.
g = acceleration due to gravity in dynes—981.
r^2 = radius of sphere squared—0.006304 cm.^{2}
ϕ = density of sphere—7.65 grams per ml.
p = density of nitrocellulose—1.65 grams per ml.
T = time of fall—10 seconds
t = temperature of nitrocellulose—24°C.

Stokes equation, $9\mu S = 2\,g\,r^2\,(\phi - p) \times T$, for d/D *very small.*

$$\text{solving} \rightarrow \mu = 0.54970 \times T$$

$$\text{Faxén wall correction factor} = 1 - 2.104\,\frac{d}{D} + 2.09\left(\frac{d}{D}\right)^3 - 0.95\left(\frac{d}{D}\right)^5$$

$$\text{or } \mu = 0.54970 \times \text{Faxén wall correction factor} \times T$$

$$\text{solving } \mu = 0.45613 \times T$$

$$= 4.56 \text{ poises at } 24°\text{C.}$$

For highly accurate determinations, a thermostatically controlled water bath is required; otherwise a temperature-correction factor can be employed (4). It is important, however, to use an accurate thermometer because each degree of rise in temperature is followed by a considerable decrease in viscosity. The nitrocellulose must, therefore, be in thermal equilibrium with the water in the bath before viscosity determinations are made. Temperature correction is calculated by the equation

$$\text{Log } V = \text{Log } V_1 + 0.0207\ (T_1 - T)$$

V = viscosity corrected to temperature T
V_1 = viscosity observed at temperature T_1

While this equation can be used for nitrocellulose in acetic acid, it may not be applicable to all the commonly used solvents. Solutions of nitrocellulose should be stored in the cold. Glacial acetic acid crystallizes at approximately 12°C.; when removed from the cold room, it melts rapidly and the solution is ready for use.

SEITZ NO. 6 FILTER

The Seitz filter No. 6 is a very satisfactory apparatus for the ultrafiltration of small quantities (100 ml.) of material. It consists of a metal delivery stem, a metal screen to support the filter medium, and a container. The usual container is of metal but one of Pyrex is satisfactory. The apparatus can be used with either positive or negative pressure.

Preparation of Membranes.—Prepare and standardize nitrocellulose (Parlodion) acetic acid solutions of varying percentage composition according to the given procedure. Pour 35 ml. of the nitrocellulose solution upon the center of a carefully leveled glass cell, 20 cm. in diameter, shielded at the sides from air currents. Solutions having a viscosity greater than 25 poises should be warmed to 30°C. before pouring. Allow the acid to evaporate at room temperature, from 21° to 25°C., for exactly two hours, after which flood the membrane rapidly with 150 ml. of ethyl alcohol, approximately 25 per cent by volume.

When the membrane is flooded initially with water the surface is rough, presumably owing, in part, to the marked interfacial tension between the water and the acetic acid. The surface tension of acetic acid at 20°C. is 27.63 dynes per cm. (5), whereas that of distilled water is 72.75 dynes per cm. The alcohol delays coagulation of the nitrocellulose and a membrane more nearly uniform in porosity is obtained. Furthermore, it is much easier to flood the nitrocellulose evenly; and the surface tension of 25-per-cent alcohol, 34.08 dynes per cm., approximates that of the acetic acid.

After thirty minutes, decant the alcohol and place the membrane and cell in running tap water overnight. Remove the membrane from the cell the following morning when maximum shrinkage has been attained. Leaving a margin of 4 cm., cut four membranes 6.1 cm. in diameter from this sheet with a steel die. Wash the membranes for forty-eight hours in at least four changes of distilled water and store in water containing sufficient sodium hypochlorite to give a final concentration of chlorine of approximately 25 p.p.m.

Place the Seitz apparatus without the membrane in a small covered pail and sterilize in the autoclave. Then, with aseptic precautions, place a membrane of the desired percentage of nitrocellulose in the sterilized filter.

When assembled, place 110 ml. of sterile distilled water in the chamber, apply a pressure of 760 mm., and collect the water in a graduate. The average time required for two 50-ml. portions of water to pass through the filter at 760-mm. pressure is designated as the "water flow." After the water flow has been observed, place 100 ml. or less of the material to be filtered in the chamber and replace the graduate with a sterile bottle. Begin filtration.

ALUMINA THIMBLE ULTRAFILTER

Volumes of material varying from a few hundred milliliters to about 4 liters are usually filtered through the alumina thimble ultrafilters; however, as many as 20 liters have been filtered through one thimble. The apparatus is so designed that the material is automatically fed into the filter chamber under conditions that exclude contamination.

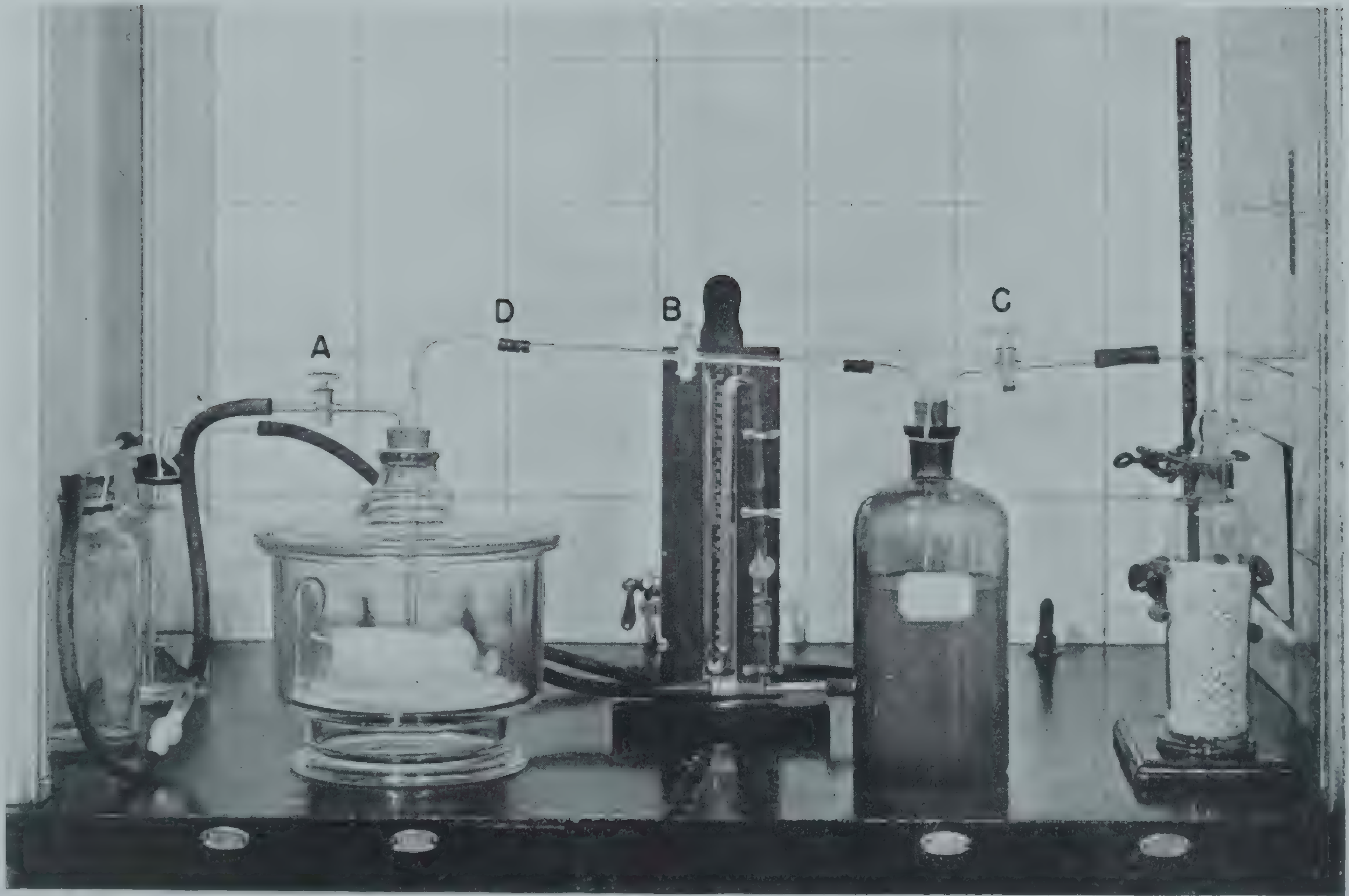

FIG. 70. APPARATUS FOR COATING PAPERS AND THIMBLES WITH NITROCELLULOSE BY VACUUM IMPREGNATION

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Equipment.—Coarse alumina thimbles are used (127 by 45 mm. with a 2-mm. wall). (Norton Company, Worcester, Massachusetts.) The thimbles are coated by the vacuum-impregnation method. The apparatus (see Fig. 70) consists of three principal parts; a 200-mm. Pyrex desiccator with a 2-holed rubber stopper inserted in the opening of the cover, a crystallizing dish in which the thimbles to be impregnated are placed, and a tared 2000-ml. amber glass bottle with a 2-holed cork stopper containing the nitrocellulose solution. A glass rod is bent so that it will rest on the edge of the crystallizing dish and extend inward to the bottom for about 12 cm. at a height that will permit the thimble on it without touching the dish.

Procedure.—Connect the desiccator containing the vessel in which the thimbles are suspended by 7-mm. glass tubing *D* extending from the bottom of the inner vessel to the bottom of the stock bottle. Insert glass stopcock *B* in the connecting tubing midway between the bottle and the desiccator. This stopcock is lubricated with a small amount of Silicone grease. When ready for use, open stockcocks *A* and *C* and close *B*. Evacuate the desiccator and contents to a pressure of approximately 80 mm.; maintain for about fifteen minutes. Open stopcock *B* and allow the nitrocellulose solution to flow into the dish until the thimbles are immersed. Again maintain a pressure of approximately 80 mm. for about thirty minutes or until only a very few air bubbles rise from the thimbles. Close stopcock *A*, disconnect the vacuum tubing, and attach a calcium chloride drying tower. Slowly open stopcock *A* and allow air to enter the desiccator until atmospheric pressure is restored. This procedure forces the nitrocellulose into the pores of the thimbles. After about thirty minutes the thimble is removed from the nitrocellulose by grasping it not more than 1 cm. from the open end and slipping it off the glass rod. Thin rubber gloves are necessary to avoid burning the hands. A convenient holder is made by inserting a short piece of 6-mm. glass rod into a hole in a 1½-inch cork, flush with the cork at the bottom and extending about 3 inches above the top. The opposite sides of the cork are cut away to a depth of one-half of an inch and to a width of three-quarters of an inch. The cork is then placed in the mouth of the thimble and the excess nitrocellulose allowed to drain; then the thimble and cork are plunged into cold tap water or 25-per-cent ethyl alcohol and suspended by a string attached to the glass rod. Initial solidification occurs in about ten minutes in water or thirty minutes in 25-per-cent ethyl alcohol; the cork is removed before it becomes swollen, and the thimble is inverted in tap water. After seventy-two hours in frequent changes of tap water, the membranes are usually free from acid.

The thimbles are mounted in discarded metal holders of 10-inch Mandler filter candles, so altered that the thimble can be fitted into a small slot about 8 mm. deep and 4 mm. wide. A mixture of melted paraffin and beeswax (melting point 56°–58°C.) is poured into this slot to overflow and to cover the previously warmed metal surface; the thimble is inserted; after cooling, a rigid, nonabsorbent, liquid-tight joint is formed. The coated thimble is locked into a $5\frac{5}{8}$- by $2\frac{3}{4}$-inch Pyrex mantle with standard Mandler washers and nuts. To permit the escape of air, a second hole is drilled in the bottom of the mantle. The filter is then placed in filtered tap water and connected by pressure tubing to a 4-liter suction flask which is evacuated to about 80-mm. pressure. The amount of water collected in the suction flask in one hour is designated as the "rate of flow." After this has been determined, the filter is removed from the Pyrex mantle and reassembled for the filtration. The apparatus is illustrated in figure 71.

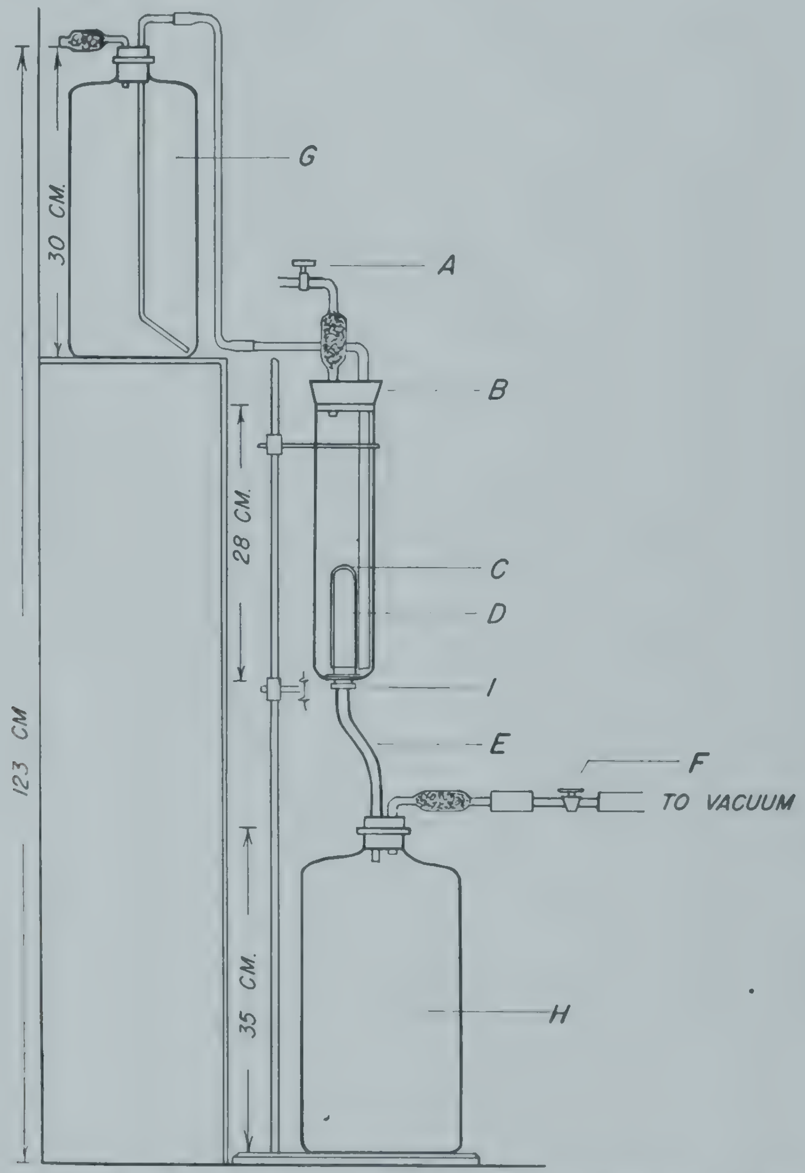

FIG. 71. APPARATUS FOR THE RAPID ULTRAFILTRATION OF LARGE QUANTITIES

By the use of a vacuum manifold, a series of 10 ultrafilter units of this type can be operated at one time.

All parts, except the nitrocellulose thimble, are autoclaved, and the entire apparatus, including the thimble, is assembled with aseptic precautions. Place in position *G* a bottle containing 2500 ml. of sterile distilled water to which sufficient 3-per-cent sodium hypochlorite solution has been added to give a final concentration of chlorine (using the ortho-tolidine reagent (6)) of approximately 25 p.p.m. This chlorinated water is drawn through the apparatus to sterilize the pores of the membrane. While the chlorine solution does not appear to exert any deleterious effect upon the nitrocellulose, it does eventually attack the rubber connections. When approximately 1500 ml. have filtered through the membrane, siphon the remainder of the chlorine water from the cylinder into *G* and substitute a bottle of sterile distilled water in position *G*. The traces of chlorine in the cylinder and in the pores of the membrane are removed by intermittent washing. Care must be exercised not to allow the nitrocellulose membrane to become dry.

After sterilization of the membrane is accomplished, place the material to be ultrafiltered in position *G*. This elevated position has two advantages: the material is introduced into the cylinder with the aid of gravity; and a positive pressure is maintained in the cylinder, thereby preventing the entrance of contaminants. Siphon from *G* into Pyrex mantle *C* to within approximately 5 cm. from the bottom of stopper *B*, close pinchcock *A* and evacuate bottle *H* to about 80 mm. of mercury. Close glass stopcock *F* to maintain vacuum for overnight filtration.

The design of the apparatus is such that very efficient washing of the residue can be accomplished by substituting a bottle of sterile distilled water at *G*. If the washed residue is not too viscous, a concentration to approximately 50 ml. can be obtained.

Cleaning of Thimble.—It is of the utmost importance that the thimbles be thoroughly cleaned; otherwise, it is difficult to obtain uniform porosity. The following procedure should be used.

Place the filter in hot water for a few minutes to soften the wax so that the thimble can be separated from the holder. Allow to dry in the air for twenty-four hours and remove by hand as much of the nitrocellulose as possible. Place the thimble in the muffle furnace and ash the remainder of the nitrocellulose. Cool, place in a 5-per-cent hydrochloric acid solution, and heat at 80°C. for about one-half hour. Cool, decant the acid solution, and wash the thimbles until they are free from acid. Dry in an oven at 110°C. Store in a desiccator over calcium chloride.

SEITZ ULTRAFILTER

The Seitz ultrafiltration apparatus consists of two parts: the sheet filter and a pressure tank connected to the filter by means of heavy rubber tubing. Sheet Filter No. 20 is used as an ultrafilter for large volumes of material from which a sizable residue will be obtained. The number of membranes can be varied in odd numbers from 1 to 9, each having an effective filtering area of about 360 square centimeters or 56 square inches.

Because of the large area of the membranes, vacuum impregnation is not practicable and the following technic has been adopted.

Equipment.—The membranes are cast in glass cells, 8 inches square, made from 2 pieces of plate glass 12 inches square and one-quarter inch thick. An 8-inch square is cut in the center of one plate and the 2 plates cemented together. A satisfactory cement is made by heating together one part of linseed oil and 25 parts of rosin, grade X. The glass plates are then warmed and the cement, while fluid, is added to the base plate. They are pressed together and allowed to cool slowly. The leveling table for supporting the cell is of metal, 12 inches in diameter. (Harvard Apparatus Company, Dover, Massachusetts.) Care must be taken in leveling the table to secure equal distribution of the nitrocellulose over the base of the cell.

Procedure.—Pour 50 ml. of nitrocellulose of the desired percentage and viscosity into the center of the cell. Solutions of low viscosity, 2.5 to 10 poises, will spread evenly over a leveled plate, but with those of higher viscosity more care must be exercised. Allow the nitrocellulose to become immobilized. With solutions of low viscosity and percentage composition two hours are sufficient; less time is required for the more concentrated and viscous solutions. Flood the cell with 25-per-cent ethyl alcohol for thirty minutes. Decant the alcohol and place the cell in tap water. In about two hours detach the membrane from the cell and wash in frequent changes of tap water until free from acetic acid. This requires three or four days.

Filtration.—In assembling the Seitz ultrafilter, No. 20, the following procedure has been adopted: a K-2 clarifying sheet that has been sterilized in a metal holder to prevent warping is placed directly in front of the outlet screen. The membrane is put next to the K-2 sheet. The apparatus is tightened and ready for testing.

Pour several liters of chlorinated tap water (50 p.p.m.) into the pressure tank and maintain a definite pressure (5 pounds per square inch) by a reducing valve; the amount of water filtering through in one hour is designated as the "rate of flow."

The material to be filtered is placed in the pressure tank which is sterilized by chemical means or by autoclaving. In large-scale ultrafiltration, the material generally contains a preservative and, as the time of ultrafiltration is usually short, no difficulty should be encountered from contaminants.

The maximum pressure employed in this type of apparatus should not exceed 20 pounds per square inch.

PREPARATION OF SPECIAL MEMBRANES

To prepare membranes for the fritted glass funnels or alundum crucibles used for quantitative ultrafiltration (see p. 110), fill the funnel or crucible with an appropriate nitrocellulose solution and let stand for thirty minutes. Decant, and let drain for about 15 seconds. Subsequent treatment is similar to that used for thimbles. Experience is required to obtain a suitable thickness of the membrane for a given purpose. The speed with which distilled water passes through it serves as a rough indication of the character of the membrane.

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CHAPTER 72

PREPARATION OF DIAGNOSTIC, PROPHYLACTIC, AND THERAPEUTIC PRODUCTS FOR DISTRIBUTION

The preparation for distribution of products for prophylactic and therapeutic use, and of certain sera and reagents for diagnostic purposes includes the bottling, labeling, boxing, storage, and transfer of the products to the shipping room. This work is carried on by the "filling and boxing group," certain members of which also assist in procedures connected with the tests for sterility and harmlessness. The head of the group is directly responsible to the Assistant Director in Charge of the Antitoxin, Serum, and Vaccine Laboratories.

The various preparations are filled in rooms designed for the purpose. Products that have been dispensed are stored in separate cold rooms at from 4° to 6°C. They are kept at room temperature, never in direct sunlight, only as long as necessary for the procedures of dispensing, labeling, and boxing.

DISPENSING OF PRODUCTS

All products are dispensed with rigidly aseptic precautions in glass-enclosed rooms equipped with air filters, ultraviolet lights, two in each room, and shadow-proof lighting. An anteroom with a swinging door leading to the filling room and a sliding door to the entrance hall provides space for receiving and storing supplies and equipment and for their preliminary preparation. As the workers enter the filling room they step on a mat soaked with disinfectant.

All flat surfaces and apparatus in the room are wiped with a disinfectant as are the outer surfaces of all utensils before they are taken in. Siphons, burettes, vials, stoppers, rubber-tubing connections, and the gowns and caps worn by the workers are sterilized by autoclaving the day before use. Rubber gloves are worn throughout the filling procedure.

The dispensing apparatus which is assembled with strictly aseptic technic consists of a graduated burette (25 or 50 ml.) connected by rubber tubing with a siphon in the bottle of material to be dispensed. This in turn is connected with a guard bottle partly filled with 5-per-cent phenol solution. The tip of the burette is protected by a glass bell.

Various automatic filling devices have been considered, but as yet none has been found sufficiently satisfactory to adopt for general use. For dispensing precipitated diphtheria toxoid, the following procedure has assured the distribution of uniform amounts of precipitate.

Connect the positive pressure system regulated by the aid of a manometer (A) with a phenolized glass-wool guard (B) attached to the inlet tube of a guard bottle (C) partly filled with 5-per-cent phenol solution. The guard (B) consists of a large pipette bulb packed with glass wool saturated with a mixture of glycerol and 5-per-cent phenol. The air outlet (D) is a sterile cotton guard, the outer end of which is covered between fillings. The air intake of the toxoid bottle is a glass tube (E) tied to the siphon (F) and extending parallel to it as far as the bend. At this point there is inserted for flexibility a piece of rubber tubing (G) to which is attached a glass bulb with small perforations (H). When the parts are correctly adjusted and held at the proper angle, the siphon (F) and air tube (E) lie side by side and do not touch the neck of the bottle as they are inserted into the toxoid which has been mixed by vigorous rotation. When the stopper is in place, the end of the siphon and the air bulb should lie near each other touching the bottom. The air intake is connected with the outlet of the special guard bottle and the usual cotton guard (J) permits egress of the air. (See Fig. 72.)

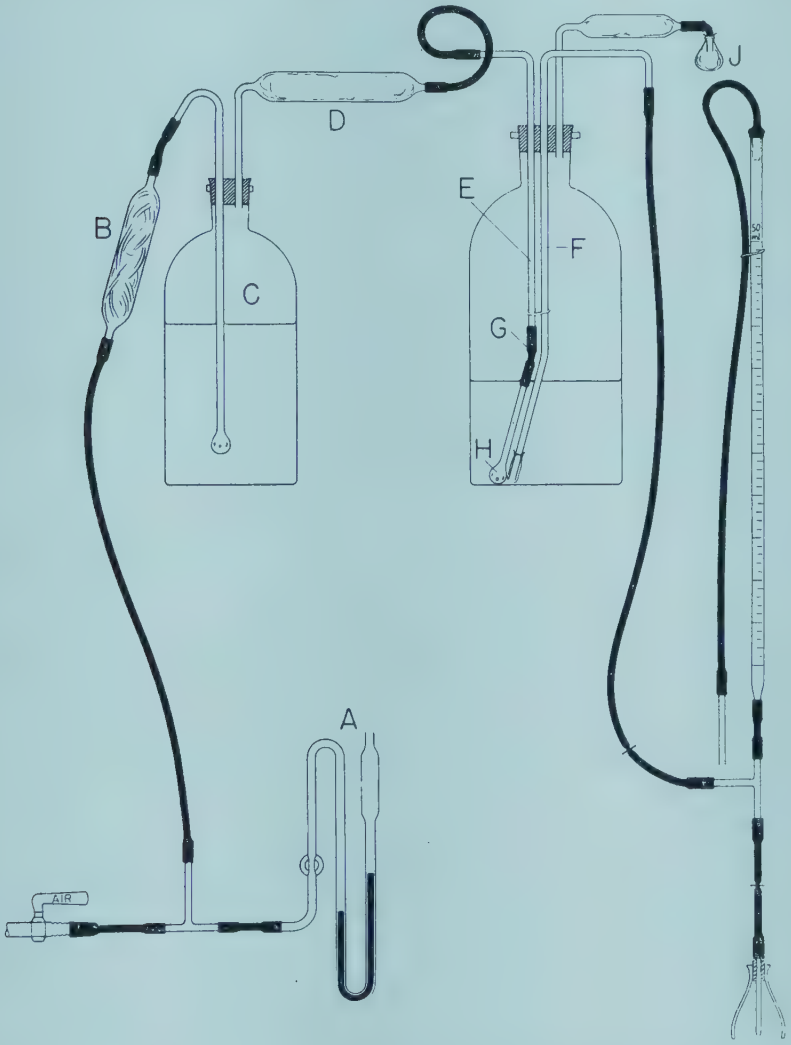

FIG. 72. APPARATUS FOR DISPENSING PRECIPITATED TOXOID

When the various parts are in place, carefully rotate the toxoid bottle again, then turn on the air pressure very slowly, watching both the manometer and the resultant bubbling until an effective agitation is obtained. Proceed with the dispensing.

Should the foam approach the stopper, carefully reduce the pressure. If there is any evidence of the precipitate's settling, rotate the bottle and carefully increase the pressure. As an extra precaution, rotate the bottle occasionally.

All preparations for human use are dispensed in vials of resistant glass made according to specifications. The necks of the different sized vials are machined with a special tool so that all diameters are the same and permit the use of a uniform stopper. The stopper is made of high-grade rubber and designed to allow convenient puncture by needle for the withdrawal of material.

Throughout all procedures careful attention to details, strict accuracy in dispensing volumes, and rigidly aseptic precautions are essential. One worker fills the vials, another inserts the stoppers. After each product is dispensed, the room and the equipment used are thoroughly cleaned.

As soon as the vials are brought from the room, they are sealed with a glycerol-glue mixture.

Mix 380 ml. distilled water, 20 ml. 0.1-per-cent phenylmercuric acetate, 160 grams gelatin, 80 ml. glycerol, and 10 ml. cresol in a double boiler and heat until completely dissolved. Skim the mixture. Allow to stand for one day before use. Keep in the double boiler and warm over a flame before applying. If the glue becomes too thick, dilute with distilled water containing a few drops of cresol. The consistency should be that of a thick sirup.

Residues of antitoxins and sera are either saved for reconcentration or disposed of.

Diagnostic sera and reagents are dispensed in high-grade glass bottles, as indicated, with either cork or rubber stoppers. The rubber stoppers are covered with paraffin, the cork with paraffin and "Gelcaps," except in the case of vials containing antisheep amboceptor when the stoppers are sealed with the glycerol-glue mixture.

As a control of accuracy in the dispensing of materials, vials, usually those containing less than 6 ml., are selected at least once a month and the contents measured by the technician in charge of the sterility tests. Select at random two bottles of material recently dispensed. Measure the contents of each in a "to contain" pipette with a capillary stem graduated in hundredths. For apparatus used in filling the pipette, see "General Instructions," p. 619. Draw all of the material into the pipette and take the reading quickly before air bubbles enter.

Tests for Sterility and Harmlessness.—Both cultural and animal tests are made on all preparations distributed for use in persons, except that with diluted diphtheria toxin for intracutaneous tests of susceptibility the animal tests are omitted; only cultural tests are made on preparations for diagnostic use.

PREPARATION FOR DISTRIBUTION

As soon as the tests are satisfactorily completed, the vials are labeled and in the case of products for human use and of certain diagnostic reagents are boxed individually. Preparations such as diphtheria toxoid and typhoid vaccine may under certain conditions be distributed in packages of twenty vials. A circular of directions is always enclosed and when information on clinical results is important, as with antitoxins, sera, and certain vaccines, a report form also.

Printed Matter.—General information is printed on the labels for each product and space is allowed for inserting special data, such as lot number, return date, unit content, and number of milliliters. Report forms and circulars of directions are printed except those for the few products that are not distributed generally. A hand press is used for printing the special data on labels, report forms, and boxes.

Labeling and Boxing.—With few exceptions, vials are labeled by machine. Vials of 1-ml. capacity, since they are too small to be used with the present machine, are labeled by hand. At the time of boxing, the vials of dispensed material are inspected and those found to have cracks or leaky stoppers or those in which the appearance of the material is not satisfactory are marked as defective and either discarded or if of any value, saved. White cardboard collapsible cartons are used. The data on the carton are compared with those on the label.

Release for Distribution.—Material is released for distribution by the head of the group that prepared it only after tests of sterility and harmlessness are satisfactorily completed. One or more bottles of every lot are retained for reference.

CHAPTER 73

STERILITY TESTS OF PROPHYLACTIC, THERAPEUTIC, AND OTHER PREPARATIONS

The sterility of all prophylactic, therapeutic, and other preparations is controlled by cultural tests of material in the process of preparation and of the products when dispensed for distribution. The harmlessness of products for human use is further controlled by the inoculation of animals. Material is not released for distribution unless the final cultural tests show the absence of growth during seven days and the animal tests have been satisfactory for the same period.

All sterility tests are under the immediate supervision of a specially trained and qualified technician who, assisted as required by one of the staff assigned to the final dispensing of products, inoculates the culture media for tests of material dispensed for distribution, examines all media after inoculation, injects and observes the test animals, and checks the volumes dispensed in final containers. The media for tests of products in bulk are inoculated by the staff who prepare and filter the material.

CULTURAL TESTS

CULTURE MEDIA

In order to provide especially favorable conditions for the growth and detection of microorganisms that may be present, two kinds of media have been selected for routine tests of the various preparations. The first, "sterility-test broth," consists of horse-meat infusion medium containing 0.1 per cent of glucose (*F3B*). The second, semisolid meat-infusion agar (Hitchens), has as its base the same broth medium to which 0.12 per cent of agar is added (*F32B*).

In maintaining anaerobic conditions in sterility-test broth, a layer of paraffin mineral oil mixture (*F79*), which is solid at room and semisolid at incubator temperature, has been found effective. The physical conditions resulting from the small percentage of agar in Hitchens's medium provide an exceptionally favorable environment for both aerobic and anaerobic growth; colony formation may be observed and the extent of bacterial contamination may to some degree be gauged. The medium is heated in streaming steam at 98°–100°C. for one-half hour on the day of inoculation to expel oxygen and insure a uniform consistency. After removal from the sterilizer the containers are held at 55°C. until about one hour before inoculation. Some flocculation usually occurs during storage; lots in which it is excessive should not be used for sterility tests.

When a lot of sterility-test broth is first used, one aerobic and one anaerobic tube, uninoculated, and two of each inoculated with sterile serum, are incubated as controls; in the case of a new lot of Hitchens's medium, one bottle, uninoculated, and two inoculated with sterile serum are tested. These control tests may be repeated from time to time if advisable.

Quantity of Inoculum

To avoid the danger of inhibiting the growth of bacteria, the concentration of preservative in the medium after inoculation is limited in the case of phenoloid preservative to not more than 0.01 per cent; in the case of organic mercury compounds, the limit is much lower because of their greater bacteriostatic activity; for example, with 'Merthiolate' it is less than 0.0002 per cent. In general, it is desirable to test the maximum volume of material possible within these limits. Since, however, an excessive quantity of serum or other material may give rise to a confusing precipitate or possibly inhibit the growth of certain microorganisms, the volume of inoculum is limited even when the material contains little or no preservative. For kind and content of preservative used in the various products and the volumes of each to be tested, see table 76. Material containing no preservative is treated on the same basis as that with 0.3 per cent of cresol.

In Sterility Test Broth.—Five-tenths of a milliliter of inoculum is added to a tube containing 20 ml. of broth except when the phenoloid preservative content of the material is greater than 0.4 per cent, in which case 0.4 ml. or less is added; when organic mercury compounds are used, less than 0.4 ml. is added, in practice, usually about from 0.25 to 0.3 ml.

In Hitchens's Medium.—The volumes of inoculum that may be added to various volumes of Hitchens's medium are given in table 77.

The Tests

Material in Bulk.—Products in bulk intended for human use are tested in both broth and Hitchens's medium, except unfiltered sera, certain concentrated vaccines, and toxoids in preparation. These and all other products are tested only in broth. If a bottle is opened subsequently, the test in broth is repeated. For further details and for exceptions, see table 76.

Material from Burette.—In the case of products for human use, 2 tubes each of aerobic and anaerobic broth are inoculated directly with material from the burette at the beginning and at the end of a filling.

Material from Final Containers (for Human Use).—The sterility of all material for human use is tested in both broth and Hitchens's medium. The final containers to be tested are selected by the worker in charge of sterility tests. The number selected and the volume of inoculum from

TABLE 76

Sterility tests of products in bulk

PRODUCT	PRESERVATIVE, PER CENT CONCENTRATION	WHEN TAKEN	VOLUME OF INOCULUM FROM CONTAINER OF			
			Less than 1 liter		More than 1 liter	
			In broth	In Hitchens's medium	In broth	In Hitchens's medium
			ml.	*ml.*	*ml.*	*ml.*
Serum*	None	On receipt	1	0	1	0
Toxin for use in animals	Cresol, 0.25%	After filtration	1	0	1†	0
Serum for therapeutic use	Cresol, 0.3%	After filtration	1	2	2	8
Serum for diagnostic use	Cresol, 0.3%	After filtration*	1‡	0	2	8
Bacterial vaccine§ concentrated	Cresol, 0.3%	1 day after addition of preservative	1‖	0	2	0
Bacterial vaccine§ concentrated	'Merthiolate,' 0.02%	After pooling	1‖	0	2	0
Bacterial vaccine diluted	Cresol, 0.3%	After dilution	1	2	2	8
Bacterial vaccine diluted	'Merthiolate,' 0.01%	After dilution	1	2	2	6
Tuberculin for use in persons	Cresol, 0.3%	After filtration	1	2	2	8
Toxin for use in persons	Phenol, 0.4%	After dilution	1	2	2	6
Toxin for use in persons	Phenol, 0.5%	After filtration	1	2	2	6
Toxin for use in animals	Phenol, 0.5%	After filtration	1	0	1†	0
Toxin for use in persons	'Merthiolate,' 0.005%	After filtration	1	2	2	6
Serum for therapeutic use	'Merthiolate,' 0.01%	After filtration	1	2	2	6
Serum for diagnostic use	'Merthiolate,' 0.01%	After filtration*	1‡	0	2	6
Toxoid¶	'Merthiolate,' 0.01%	After addition of preservative	1	2	2	6
Serum for diagnostic use	Glycerol, 50.0%	After addition of preservative	1‡	0	2	8
Serum for therapeutic** use	'Merthiolate,' 0.005% + Phenol, 0.2%	After filtration	1	2	2	6
Globulin solution	'Merthiolate,' 0.005% + Phenol, 0.2%	After filtration	1	2	2	6
Toxin diluted for use in persons	Borate buffer	After filtration	1	2	2	8

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* When a lot of serum consists of less than 250 ml., test on receipt 0.5 ml. in Hitchens's medium and omit the broth test. Diagnostic sera that are not filtered are tested similarly.

† If a lot consists of less than 4 bottles, or if the toxin is filtered into 8 liter bottles, test 2 ml. of inoculum.

‡ When the volume is less than 500 ml., test 1 ml.; when from 500 to 1000 ml., 2 ml

§ For additional tests see section on preparation and standardization of product

‖ At the first examination, if the material is very cloudy, make subcultures from each tube of broth by transferring 0.5 ml. to each of two similar tubes.

¶ After incubation but before 'Merthiolate' is added, test 1 ml. in broth.

** After preliminary filtration of immune rabbit serum for therapeutic use make following tests: From containers of less than 1 liter, 1 ml. in broth (*F8B*) and 1 ml in Hitchens's medium (*F32B*); from containers of more than 1 liter, 1 ml in broth and 2 ml. in Hitchens's medium.

each are based on a sliding scale and are determined by the number of bottles dispensed and the amount in each. See table 78. For the tests in broth, 2 ml. from each of 2 bottles selected at random from the first hundred, and from one bottle in each succeeding hundred are added to the broth. If the bottles contain more than 2 ml., additional material from each is tested in Hitchens's medium. An exception is made when products highly diluted with salt solution, such as precipitated diphtheria toxoids and bacterial vaccines, are dispensed in volumes of more than 2 ml. In such cases, the usual number of bottles are tested in broth only. From every filling, moreover, one bottle from each hundred up to four hundred is selected for tests in Hitchens's medium; when more than four hundred bottles are filled, one is selected from the first hundred, one from the next to the last, and one from each of two other hundreds.

TABLE 77

Volumes of inoculum permissible in given volumes of Hitchens's medium

HITCHENS'S MEDIUM	INOCULUM WITH A PHENOLOID PRESERVATIVE CONTENT OF			INOCULUM WITH 'MERTHIOLATE'* PRESERVATIVE CONTENT OF
	0.3 per cent	0.4 per cent	0.5 per cent	0.01 per cent or 0.005 per cent
ml.	*ml.*	*ml.*	*ml.*	*ml.*
20	0.7 or less	0.5 or less	0.4 or less	0.3 or less
40	0.8 to 1.3	0.6 to 1	0.5 to 0.8	0.4 to 0.5
80	1.4 to 2.7	1.1 to 2	0.9 to 1.6	0.6 to 1.2
160	2.8 to 5.5	2.1 to 4	1.7 to 3.2	1.3 to 3.0

* Products containing both 'Merthiolate' and phenol or organic mercury compounds other than 'Merthiolate' are tested similarly.

For tests in broth, the 2 ml. of inoculum from a bottle are divided among 2 tubes each of aerobic and anaerobic broth, 0.5 ml. being added to each. If the material to be tested contains phenoloid preservative in a concentration of more than 0.4 per cent, 'Merthiolate' or other organic mercury compounds, the inoculum is distributed among 3 tubes each of aerobic and anaerobic broth. The amount of the remaining material tested in Hitchens's medium from these bottles depends upon the total number of bottles filled. When 100 bottles or less are filled, up to 5 ml. of the material are added to sufficient Hitchens's medium to give a final concentration of not more than the prescribed limits of preservative; when from 101 to 500 bottles, 3 ml.; when from 501 to 800, 2 ml.; and when from 801 to 1000, 1 ml. If over 1000 bottles are filled, the additional test in Hitchens's medium is omitted from the bottles selected for the tests in broth. If 5 ml. of material containing 0.3 per cent of cresol are to be tested, add the inoculum to 160 ml. of Hitchens's medium in one container; if the preservative is 0.01-per-cent 'Merthiolate,' divide the 5 ml. between two 160-ml. amounts of Hitchens's medium. For further details on the volumes of Hitchens's medium required for the above tests, see table 77.

In the case of containers selected for tests in Hitchens's medium only, the contents of each bottle, up to 5 ml., are added to sufficient medium to give a final concentration of not more than the prescribed limits of preservative.

TABLE 78

Sterility tests of products in final containers (for human use)

NUMBER OF BOTTLES FILLED*	BROTH AND HITCHENS'S MEDIUM, TESTS FROM SAME BOTTLES				HITCHENS'S MEDIUM, TESTS FROM SEPARATE BOTTLES	
	Number of bottles	Volume inoculum from each bottle			Number of bottles	Volume inoculum from each bottle Contents to:
		Broth	Hitchens's medium† Balance contents to:	Total contents to:		
		ml.	*ml.*	*ml.*		*ml.*
100 or less	2	2	5	7	1	5
101 to 200	3	2	3	5	2	5
201 to 300	4	2	3	5	3	5
301 to 400	5	2	3	5	4	5
401 to 500	6	2	3	5	4	5
501 to 600	7	2	2	4	4	5
601 to 700	8	2	2	4	4	5
701 to 800	9	2	2	4	4	5
801 to 900	10	2	1	3	4	5
901 to 1,000	11	2	1	3	4	5
More than 1,000	One additional bottle from each hundred	2	0	2	4	5

* For procedure in the case of very small fillings or when the volume is less than 2 ml., see text, p. 832.

† For exception made when products highly diluted with salt solution and to be administered in doses of 1 ml. or less are dispensed in volumes of more than 2 ml., see text, p. 831.

When less than 25 bottles are filled or from 25 to 50 bottles containing more than 12 ml. of material, only 2 bottles are selected for the test. Two milliliters from each are added to the broth and the remaining material, up to 10 ml., to Hitchens's medium.

When material is dispensed in less than 2-ml. volumes, only 1 ml. is inoculated from each bottle selected; when in less than 1-ml. volumes, twice the usual number of bottles are selected, that is, 2 pairs of 2 adjacent bottles each from the first hundred, and one pair from each succeeding hundred.

When material is dispensed in more than one volume, at least one bottle of each size is selected for tests.

Material from Final Containers (for Diagnostic Use).—Material for diagnostic use is tested in broth and in Hitchens's medium. When less than 10 bottles are filled, one bottle only is selected; when from 10 to 50, 2; when from 51 to 100, 3; and when more than 100, 3 for tests in broth and 2 for tests in Hitchens's medium, one from the first hundred and one from the remaining number, or from next to the last hundred if there are over 200 bottles.

Tests in broth are omitted when less than 10 bottles are filled. One milliliter of inoculum is tested in broth from one bottle when from 10 to 50 are filled; from each of 2 bottles when 51 to 100; and from each of the bottles selected for tests in broth, when more than 100.

For tests in Hitchens's medium, 1 ml. of inoculum is tested from the bottle selected when less than 10 are filled; from the second bottle when 10 to 50; from the third when 51 to 100; and from each of the bottles selected for tests in Hitchens's medium when more than 100.

When material is dispensed in a single container for special distribution, the following tests are made. If the total amount is 10 ml. or less, inoculate one tube each of aerobic and of anaerobic sterility-test broth from the pipette used to transfer the material. Transfer 0.5 ml. from the bottle to Hitchens's medium. If the volume is from 11 to 25 ml., 1.5 ml. from the bottle is tested, 1 ml. in broth and 0.5 ml. in Hitchens's medium; if from 26 to 500 ml., 1 ml. in broth and 1 ml. in Hitchens's medium; if from 501 to 1000 ml., 1 ml. in broth, 2 ml. in Hitchens's medium. If it is particularly desirable that a bottle be as full as possible, as in the case of diphtheria toxin, 2 bottles may be filled and the material for the tests taken from the second.

Inoculation of Culture Media

The procedures connected with the inoculation of the media must be carried on with rigidly aseptic precautions in a special room. The containers of pipettes must have been freshly sterilized and not opened since sterilization. A fresh pipette is used for each bottle and every time a bottle is opened.

Take every precaution to avoid mixing sets of tubes or bottles. As soon as a set is inoculated, band the tubes together and attach a sterility-test record slip. Attach a similar slip to each container of Hitchens's medium.

Incubation and Examination of Inoculated Media

Incubate the inoculated media at 34°–36°C. for seven days. Examine the tubes of broth on the second, fourth, and seventh days and record the results. Examine the inoculated Hitchens's medium daily except Sundays and holidays. Record the results in the permanent record book only on the second, fourth, and seventh days except when contaminating microorganisms are found or suspected. When a Sunday or a holiday intervenes, postpone the examination for one day. If they fall on successive days, examine all inoculated media on the preceding and following days.

To avoid unnecessary cooling, remove only a few baskets of media from the incubator at one time. Make examinations in front of a window or fluorescent light. Examine one bottle of Hitchens's medium and record the results before proceeding to the examination of another. In the same way, examine only one set of tubes at a time. After observing the appearance of the broth, rotate the tubes gently to detect any precipitate or sediment that would suggest contamination. Do not rotate containers of Hitchens's medium. Mark any suspected container and observe with special care at the next examination. When the test is completed, before removing the record slip and entering the necessary data on it, record in the ledger the results of the final examination of each set of tubes and each bottle. Examine further the medium in each container in which contamination is suspected. When it is necessary to remove a tube from a set, mark it plainly with the name and lot number of the product, together with any other data essential for its identification. Do not remove the slip from the remainder of the set.

Since the examination of stained preparations of material that shows slight indication of contamination is usually indeterminate, make cultural tests at once. To one tube of deep 0.2-per-cent glucose agar (*F35A*), melted and cooled to about 40°C., add 0.5 ml. of the inoculated broth or Hitchens's medium. After inoculation of the medium, cool rapidly. Prepare a poured plate with the same medium and amount of inoculum. Incubate the deep agar tube and poured plate for five days and observe daily. With certain cloudy preparations in which growth would not be readily detected, and when contamination is suspected, make the following subcultures in addition to the tube and poured plate. From a container of Hitchens's medium, subculture to one tube each of aerobic and anaerobic sterility-test broth; from tubes of sterility-test broth, subculture to the same media, namely from aerobic sterility-test broth to aerobic, and from anaerobic sterility-test broth to anaerobic, and incubate for seven days.

When bacterial growth is obvious in inoculated medium, remove and mark the container as previously indicated. Examine a slide preparation stained by Gram's method and record the type of microorganism found. Should the presence of sporeforming microorganisms be suggested, examine a second slide preparation stained with steaming carbolfuchsin. Retain the culture in the cold room until the test is completed. Should the slide preparations fail to show bacteria, make subcultures as above. If growth is present in several containers, make slide preparations from at least two, always from both kinds of media if they are contaminated.

Retests

Retests are made when the number of containers in which growth develops is so limited that there is doubt as to whether the microorganisms gained entrance at the time of the inoculation of the medium or were present originally in the material tested.

In the case of material in bulk, if the sterility tests indicate slight contamination in one bottle, the tests on that bottle are repeated; if in two or more bottles, the tests of the entire lot are repeated. If growth develops in one or more containers in the retest, the material is treated as contaminated.

Contaminated lots of sera and toxins are filtered as soon as possible. If the extent or nature of the contamination is such as to raise any doubt as to the suitability of the product for use, the Assistant Director in charge of the Antitoxin, Serum, and Vaccine Laboratories is consulted in regard to the disposition of the material. Contaminated bacterial vaccines and precipitated diphtheria toxoid are always discarded.

With dispensed material, repeat the entire test if growth has occurred in any container. Should a retest also indicate the possible presence of contaminating microorganisms, a second retest may be made in special instances. The presence of the same microorganism in the original test and the retest is considered evidence that the material is contaminated. Certain exceptions may be allowed, such as a partial retest when "accidental contamination," has presumably occurred.

No material is released for distribution unless in the final test all containers have remained sterile.

ANIMAL TESTS

In order further to insure the harmlessness of all products for human use, material from the final containers is injected into animals. A guinea pig and a mouse are injected with material from each lot and kept under observation for seven days. Certain exceptions are made: with salt solution distributed with antipneumococcus serum, the mouse test is omitted; with small lots of less than 200 ml. of special products, such as human serum, the guinea pig test; with diluted diphtheria toxin for intracutaneous tests, both animal tests are omitted.

Mice: Healthy white mice weighing not less than 20 grams and that have not been used previously are selected. After injection, they are kept separately.

Guinea pigs: Healthy guinea pigs weighing not less than 300 grams, preferably over 350 grams, and that have not been used previously are selected. After injection, not more than six are kept together.

Injection and Dosage. Observation of Animals.—Make injections subcutaneously. For technic, see "Use of Experimental and Test Animals," p. 77. Inject 3 ml. of the test material into the guinea pig and 1 ml. into the mouse, with the following exceptions: for vaccines, inject 1.5 and 0.5 ml. respectively; for tuberculin and any other product containing 50 per cent of glycerol, 0.5 and 0.25 ml.; for sodium citrate solution, 3 ml. into the guinea pig and 1 ml. of a 1:15 dilution into the mouse.

Keep the mice under observation for at least one-half hour after injection. If a mouse shows more than the slight or even moderately severe reaction that may be expected from phenoloid preservative or does not recover promptly, notify the staff who prepared the material, and repeat the test. Examine all animals daily at 9:00 a.m. If any show evidence of illness, re-examine at 5:00 p.m. The hours are optional on Sundays and holidays Weigh all guinea pigs on the fourth day; any that appear to be losing weight, daily. Watch for general symptoms, including any indication of paralysis, and for local reactions especially at the site of injection. Transfer to a separate cage any guinea pig that has lost considerable weight or any that appears ill. Notify the staff concerned of any unusual reaction, and at the first evidence of illness or infection isolate the animal. Unless the symptoms are slight and transient and appear to be of no significance, in which case a retest may be omitted, inoculate another animal with the same dose; inoculate two, should further control be advisable. If the condition of the animal has remained satisfactory, discharge it on the seventh day.

RECORDS AND REPORTS OF TESTS

Records of all tests are kept in bound ledgers. The data required for the permanent records are entered on the special slip that accompanies each set of tubes and each bottle of inoculated medium. A report is submitted immediately should contamination be indicated. When a test is completed, the original slips and, in the case of dispensed material, an additional slip summarizing the results are sent to the staff preparing the material.

METHODS USED IN THE ADMINISTRATIVE OFFICES

ILA M. DUTTON, *Administrative Officer*

CHAPTER 74

METHODS USED IN THE ADMINISTRATIVE OFFICES

INTRODUCTION

The general administration of the laboratory is conducted by the Laboratory Committee which is composed of the associate director, the heads of the different departments, and the administrative officer. Any change recommended in policy or standards is referred to the director. The administrative offices are responsible for the executive, secretarial, and clerical work of the laboratory; records and other activities in relation to personnel administration are also centralized here. The executive offices are subdivided into departmental units, each in charge of a secretary, who, with the secretary to the director, and the librarian, form the secretariat. The secretariat has become a unique part of the executive organization. Its members, in addition to their departmental affiliations, assist in, and are responsible to the director for maintaining the continuity of established policies. They are required to have training in some branch of biologic science as well as in the technical phases of office work, and to have a reading knowledge of either French or German. They are capable of relieving the scientific staff of all clerical, secretarial, and general administrative routine, and thus they help to unify the whole laboratory organization. The laboratory committee and the secretariat meet with the director monthly in joint session for discussion of current procedures and policies.

Orientation and Training Period for New Members of the Staff.—New appointees to the secretarial and stenographic staff are assigned for short periods to the library and the various office groups for general instruction. Similarly, for the new appointee entering the laboratory as Laboratory Helper or Junior Laboratory Technician, a period of orientation and training is provided. The first step includes learning something of the functions of the Division and its place in the public health service of the state, conferences with department heads, a tour of the buildings, and an introduction to laboratory ways through the "Laboratory Handbook Prepared for the Information of the Staff."

There follows a carefully planned training and evaluation period of five weeks or possibly more. It begins with observation, practice, and reading assignments associated with the basic laboratory procedures carried on in the Department of Media and Glassware where over sixty persons take part in the work. On satisfactory completion of this initial period the new worker moves to the Collection of Type Cultures in which instruction in essential bacteriologic technics is received and methods of isolation of pure cultures and identification of species are observed and practiced. Further specialized training, or assignment to one of the routine services for training in particular technics, depends largely on the aptitudes demonstrated by the individual and the requirements of the work. Later in-service training is provided through transfers or temporary assignments to other groups or departments of the laboratory with the aim of broadening experience and stimulating initiative.

The work of the administrative offices is divided as follows:

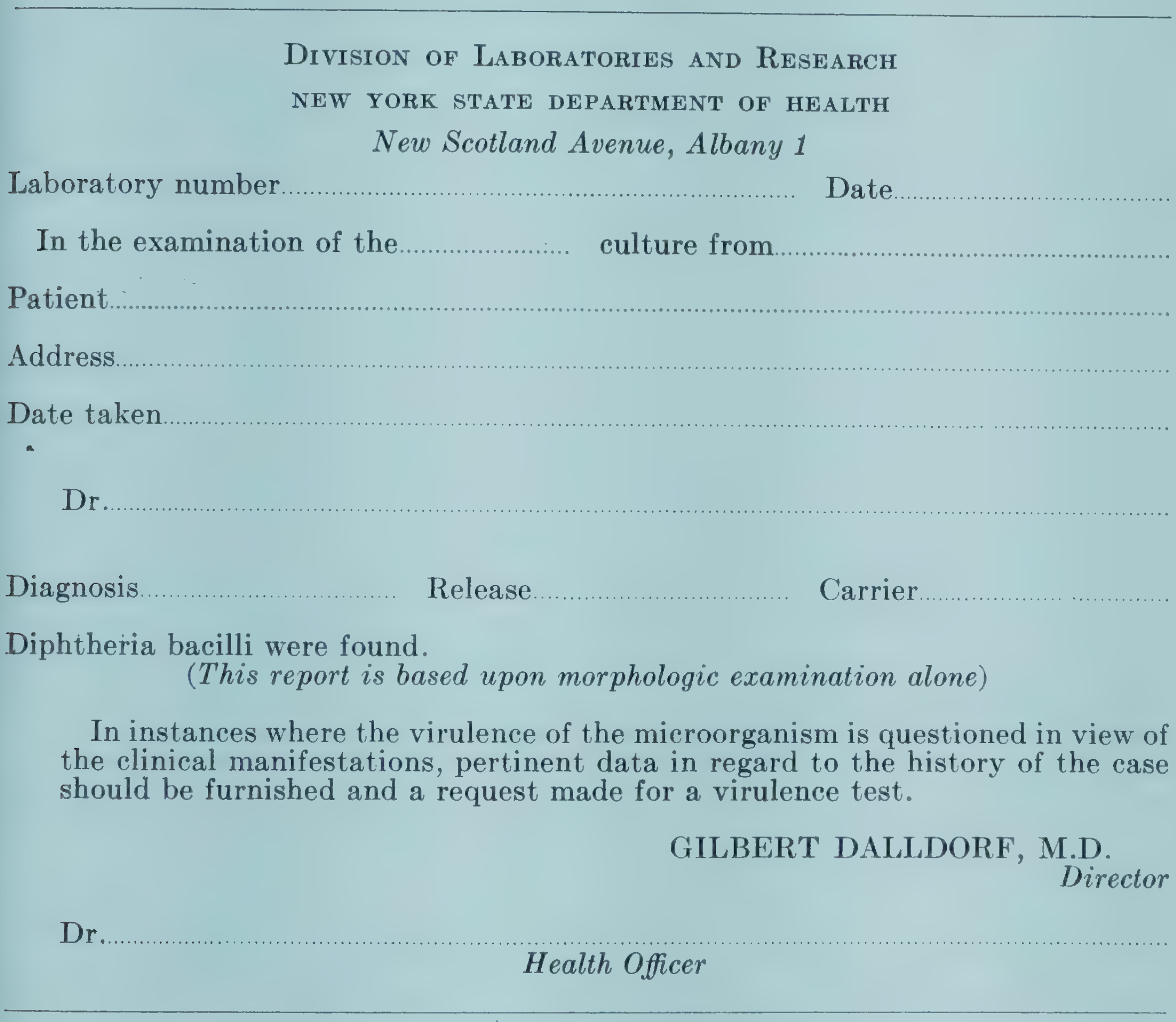
1. Recording and reporting results of examinations; administrative records and procedures connected with the approval and registration of laboratories
2. Distribution of laboratory preparations and outfits
3. Order, storage, and distribution of equipment and supplies
4. Communication; general administrative procedures.

The work has been developed and systematized in accordance with approved modern practice. Consequently, procedures common to all business offices have been omitted and only the methods and policies peculiar to this laboratory are given.

RECORDING AND REPORTING RESULTS OF EXAMINATIONS

Each specimen received in the laboratory is given an accession number and recorded in an accession book, the extent of the entry varying with the type of examination. This book serves several purposes: it is a permanent record of all specimens examined; it provides the means of determining that a prompt and accurate report has been submitted on the examination of each specimen; and it is a basis for the compilation of statistics. Typewritten reports are sent to the physician who submitted the specimen and when the examination discloses evidence of a communicable disease or of malignancy, or the results are needed for release from quarantine or observation, copies are also sent to health officials in accordance with the provisions of the Public Health Law and the Sanitary Code. Reports of the results of examinations in the sanitary and analytical chemical laboratories are sent to the Division of Sanitation, the health officer, or other designated authority, depending upon the source of the specimen and the character of the examination.

To avoid misinterpretation of the findings, results of laboratory examinations are reported only to physicians. Copies of reports are sent to hospitals and other institutions for purposes of record upon request of the physician. Standardized printed forms are used wherever possible. See figures 73–75b. Copies are made for filing only when the result is typed or when a special typewritten note has been added to the printed result. The history form received with the specimen and on which the results of the examination are written is filed and regarded as the original record of all procedures connected with the examination of a specimen. See "Appendix C," p. 879. The only exception to this practice is in the serologic tests for evidence of syphilis and tuberculosis in which the results are written directly on the accession sheet by the examiner and copied by him on the history form.



Division of Laboratories and Research
NEW YORK STATE DEPARTMENT OF HEALTH
New Scotland Avenue, Albany 1

Laboratory number .... Date ....

In the examination of the .... culture from ....

Patient ....

Address ....

Date taken ....

Dr. ....

Diagnosis .... Release .... Carrier ....

Diphtheria bacilli were found.
(*This report is based upon morphologic examination alone*)

In instances where the virulence of the microorganism is questioned in view of the clinical manifestations, pertinent data in regard to the history of the case should be furnished and a request made for a virulence test.

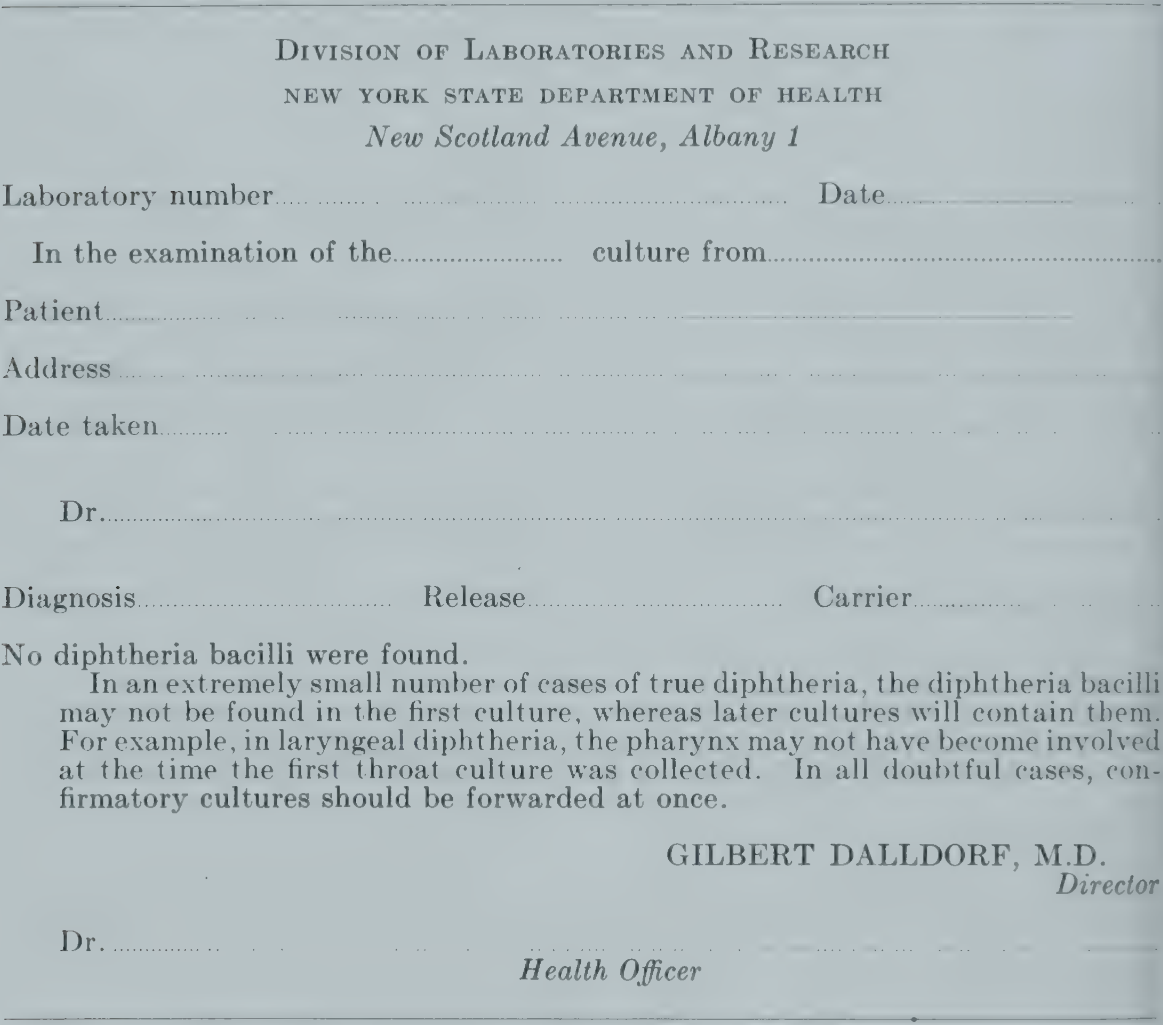
GILBERT DALLDORF, M.D.
Director

Dr. ....
Health Officer

Fig. 73. Diphtheria Report Form

CONFIDENTIAL NATURE OF RECORDS

Records of all laboratory examinations are considered confidential. Each request for information in regard to them is considered individually and every precaution taken to safeguard the interests of the patient and of the physician who submitted the specimen. Reports are generally not given to a physician other than the one submitting the specimen unless the patient signs a waiver requesting that this be done. When records are subpoenaed in litigation, before divulging any information, the witness calls attention to the confidential nature of laboratory records, and only when ordered to do so by the presiding judge does he give testimony concerning the findings. The Public Health Law and the Sanitary Code include special provisions regarding the confidential character of all records relating to cases or suspected cases of syphilis, gonorrhea, chancroid, and tuberculosis.



DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
New Scotland Avenue, Albany 1

Laboratory number........................ Date........................

In the examination of the........................ culture from........................

Patient........................

Address........................

Date taken........................

Dr........................

Diagnosis........................ Release........................ Carrier........................

No diphtheria bacilli were found.

In an extremely small number of cases of true diphtheria, the diphtheria bacilli may not be found in the first culture, whereas later cultures will contain them. For example, in laryngeal diphtheria, the pharynx may not have become involved at the time the first throat culture was collected. In all doubtful cases, confirmatory cultures should be forwarded at once.

GILBERT DALLDORF, M.D.
Director

Dr........................
Health Officer

FIG. 74. DIPHTHERIA REPORT FORM

FILING OF RECORDS

The history forms submitted by physicians with specimens for examination are classified by calendar year and type. They are then filed geographically according to the address of the physician or of the institution from which submitted, and subfiled alphabetically by patient's name or initials. The cards accompanying samples for sanitary examination are also filed according to calendar year and type, and alphabetically according to location. These records are kept permanently.

Until 1945, all history forms were filed permanently or were microfilmed. This procedure proved expensive and time-consuming in view of the limited

---

DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
New Scotland Avenue, Albany 1

Laboratory number.. Date..........................

The examination of.. from....................

Patient..

Address...

Date taken..

Dr...

Was unsatisfactory for the following reason:

(See reverse side)

GILBERT DALLDORF, M.D.
Director

Dr...
Health Officer

FIG. 75a. REPORT FORM (FRONT)

A—Received in poor condition.
- I Culture medium dried, liquefied, or contaminated.
- II Tube, jar, or slides broken.
- III Blood hemolyzed.
- IV Specimen spoiled; delayed in reaching the laboratory.
- V Insufficient material for test; specimen leaked in transit.
- VI Culture contaminated, probably by microorganisms that were present in the mouth or nasopharynx of the patient.

B—Not properly taken.
- I Insufficient material sent.
- II Culture medium not thoroughly inoculated; insufficient growth obtained.
- III Film not properly made; too thick.
- IV Film not completely dried when received.
- V Improper outfit used.

C—Result of examination indeterminate.
- I Microorganisms atypical, or their presence of doubtful significance.
- II See explanatory note.

D—Examination not made.
- I See explanatory note.

FIG. 75b. REPORT FORM (BACK)

reference and has been discontinued. The forms are now discarded annually according to the following schedule: after ten years—diphtheria, enteric disease (agglutination tests), gonorrhea, syphilis (serologic tests), tuberculosis, undulant fever, and serologic tests for evidence of other diseases such as tularemia, Rocky Mountain spotted fever, and typhus fever; after twenty years—enteric diseases (bacteriologic examinations) and miscellaneous types of examinations; after forty years—tissue examinations. The accession books have been kept in their original form but later are to be held as permanent records on microfilm. The Public Health Law requires that certain records of laboratory examinations be kept for a period to be specified by the state commissioner of health, and under the Education Law, no public records may be disposed of without the consent of the commissioner of education.

APPROVAL AND REGISTRATION OF LOCAL LABORATORIES. STATE AID

The Sanitary Code requires physicians to submit specimens from certain of the communicable diseases and of tissue removed at operation or at autopsy that require laboratory examination as an aid in the diagnosis, prevention, or treatment of disease or to determine the cause of death, to a laboratory that has been approved by the state commissioner of health for the examination of such specimens. Bacterial counts of milk for the purpose of grading, bio-assays to determine the vitamin-D content of milk, examinations to insure adequate control of the sanitary quality of public water supplies, and bacterial counts to check the cleanliness of eating, drinking, and cooking utensils must also be made in an approved laboratory. The New York State Sanitary Code is not effective in the City of New York. Laboratories there whose directors wish to make official examinations of specimens submitted from outside of the city or to act as consultants in districts where the State Sanitary Code is effective are approved by the state commissioner of health on the same basis as laboratories located elsewhere in the state.

The Public Health Law requires registration with the state department of health, or in the City of New York with the department of health of that city, of places where cultures of pathogenic microorganisms or viruses are handled, with the exception of laboratories maintained by the federal government, the state, a municipality, or a county. This law has no bearing on approval of laboratories, but provides for the compilation of a list of addresses where strains of the incitants of disease are maintained; it prohibits their sale or distribution without permission of the state commissioner of health, or in the City of New York of the New York City commissioner of health.

The Public Health Law provides for state aid to cities and counties toward the establishment and maintenance or the development of local laboratory service. See appendices *A* and *B*. The administrative procedures relating to approval, registration, and state aid are the responsibility of the offices of the diagnostic laboratories.

DISTRIBUTION OF LABORATORY PREPARATIONS AND OUTFITS

DISTRICT STATIONS

The Public Health Law provides for the establishment of district laboratory supply stations for the distribution of antitoxins, sera, vaccines, miscellaneous preparations, and outfits for the submission of specimens. It provides, further, for the appointment of a health officer or person in charge of an approved laboratory, or, when necessary, of some other competent person in the district to serve as custodian of the station. The law also permits the establishment by the custodian, with the approval of the State Department of Health, of such substations as may be necessary for the proper distribution of supplies. All stations must be maintained and operated according to the rules and regulations of the State Department of Health; the costs of maintenance are a charge upon the municipal or county funds.

It is the duty of each custodian to maintain in the main station and each substation a sufficient supply of laboratory outfits and preparations in good condition to meet ordinary demands. These supplies must be accessible to physicians at all times and under no circumstances are they to be sold.

Supply stations distribute state products (subject to stipulated restrictions with reference to certain preparations) for use in institutions and among residents of the state, and in any emergency among individuals temporarily residing in the state in which their use is likely to conserve life or health. The custodian, however, assumes responsibility for conserving the state products from waste through unnecessary or excessive distribution.

Custodians are required to keep a record of the distribution of prophylactic and therapeutic products from the district station and its substations; such record to include the name and address of each physician to whom materials are furnished, the date, the kind, the number of packages distributed, units, grams, or milliliters, lot number, number of packages returned to supply station, and whether or not reports on use of products have been received from the physicians. Reports are submitted monthly to the Division of Laboratories and Research, a copy being sent to the district state health officer. In addition, custodians report semi-annually the quantity of the various supplies on hand in each main and substation.

Each station is inspected annually by a district state health officer and if it has been maintained and operated in accordance with the rules and regulations, a certificate of satisfactory maintenance is sent to the custodian of the main station. This entitles him to receive from the municipal or county treasurer certain fees specified in the law and the actual and necessary expenses for operating the station and its substations.

All records relating to the distribution of preparations supplied by the laboratory, as well as to the perpetual inventory of products on hand, are the responsibility of the secretarial office of the antitoxin, serum, and vaccine laboratories.

PURCHASE, STORAGE, AND DISTRIBUTION OF EQUIPMENT AND SUPPLIES

PURCHASES

Standards

Standards and specifications have been established for most of the supplies and for practically all items of equipment in the laboratory. To assist the Laboratory Committee and the administrative offices in the choice of new equipment and in the proper use and maintenance of equipment and materials, there is a Committee on Equipment and Supplies composed of representatives from the several departments and the purchasing clerk.

Requisitions

Requisitions signed by the director or the heads of departments furnish the necessary authorization for purchases. Requisitions for equipment of unusual or expensive nature are submitted to the director for approval. Complete specifications must be given on all requisitions.

STORAGE AND DISTRIBUTION

As packages are opened the material is compared carefully with specifications and if satisfactory added to stock. Special apparatus is referred for approval to the member of the staff who signed the requisition. Quantities and condition of supplies received are reported by the receiving room to the office for inventory record and auditing purposes.

Material is disbursed from stock only on presentation of a requisition signed by an authorized member of the staff. Microscopes or their parts are given out only on orders approved by the director, the associate director, or the assistant directors.

Inventory

Maximum and minimum quantities have been established on all articles regularly carried in stock. In order that sufficient time may be given the purchasing group to secure quotations and place orders, the minimum quantity covers at least one month's supply. When the stock on hand reaches the minimum quantity, the maximum is purchased. Daily entries of the receipt and disbursement of stock are made on the inventory records, and balances drawn.

Auditing

The rules and regulations of the state comptroller determine the methods used in presenting bills for payment, in keeping appropriation balances, and in compiling the annual budget for the expenses of the laboratory.

GENERAL ADMINISTRATIVE PROCEDURES

COMMUNICATION

All letters except those addressed to individuals and marked "personal" are opened by the mail clerk. After the date has been stamped on the back of each piece of mail, it is taken to the director's secretary who reads it and passes it on to the administrative officer whose duty it is to read it and mark on it the name of the secretary of the department to which it is referred for attention. It is then distributed as marked. All outgoing mail is read by the administrative officer. Completed correspondence is sent to the files.

The geographic filing system is used for the majority of the laboratory records.

SERVICE-RECORD LAW

Service-record ratings for competitive civil service employees have long been required by law. Under the provisions of Chapter 859 of the Laws of 1937, the Civil Service Department has established a uniform system of service ratings for all permanent competitive and noncompetitive workers. The service record is a factor in all promotion examinations.

VOLUNTEER WORKERS

Volunteer workers with the necessary educational qualifications are accepted for training. The course offered is intended to enable students to fit themselves for technical positions in diagnostic laboratories. Special emphasis is given to training in the examination of specimens for evidence of communicable diseases.

The course covers a period of approximately two years. Students with exceptional training and qualifications may be permitted to enter the advanced phases of laboratory work and research.

VISITORS

Visitors on entering the laboratory are directed to a reception room until they can be received by the appropriate secretary or other representative. After signing the guest book, those wishing to make a tour of the building are conducted by a specially assigned guide; they are not permitted to go unescorted.

Official visitors from other states and foreign countries and qualified individuals who wish to review special subjects are given opportunity to observe different branches of the work. To facilitate this the visitor fills out a form on which he indicates under the various laboratory activities listed, those that interest him particularly. A visitor remaining for more than a day or two is expected to collaborate by preparing a critical report of his stay.

METHODS USED IN THE LIBRARY, PUBLICATIONS, AND MUSEUM DEPARTMENT

ANNA M. SEXTON, *Librarian*

CHAPTER 75

METHODS USED IN THE LIBRARY, PUBLICATIONS, AND MUSEUM DEPARTMENT

INTRODUCTION

The library department consists of two principal divisions: the publications section and the library, closely allied and jointly serving one purpose, but each with certain definite and specific functions. The museum is administered as a special collection but is developing rapidly in character and scope as a distinctive unit.

The department occupies a strategic place in the organization of the laboratory. Its members are expected by virtue of its position to be informed in regard to the routine and research activities of the several laboratory departments and to know the obligations in law of the Division as a whole, as well as the reciprocal responsibilities of the laboratory and the practicing physician or public health official. To this end the librarian is chairman of the secretariat and attends the monthly conference of the director with the laboratory committee and the secretariat to discuss laboratory procedures and policies. The librarian is also a member of the committee that plans the seminars of the laboratory staff for the discussion of intramural investigations and the review of current literature.

The library offers members of the staff the opportunity to study the scientific literature bearing on their special fields of investigation. The objectives of the laboratory are a prompt and efficient service in the diagnosis, prevention, treatment, and control of disease, and in the maintenance and control of other public sanitary services, together with essential research into the fundamental problems that underlie the work of the entire Division. There must thus be available adequate and readily accessible collections of the past and current literature in these fields and in related and supporting sciences (1). The literature of all languages is drawn upon and the library collections are constantly augmented to meet these needs. To facilitate reference, the current literature is indexed, bibliographies are compiled, abstracts and translations are made, and the attention of the staff is called to matters that may be of interest to them. The indexing service is extended to approved laboratories through the distribution monthly of mimeographed lists of selected items.

Besides books, periodicals, and pamphlets, the resources of the library include special collections of microfilms, photographs, maps, lantern slides, and photomicrographs. Motion picture films and other special exhibits prepared in the course of the work are cataloged in the library or the museum.

The publications section shares responsibility for the presentations of the laboratory through editorial advice and recommendations to members of the staff. Its aim is to establish and maintain a high standard in the publication of scientific papers and to preserve original records in connection with each investigation. Manuscripts of the reports and bulletins issued by the laboratory and of all scientific papers are filed in what is known as the "scientific file"; other items of value are incorporated in the museum.

The staff of the library department consists of a librarian, two assistant librarians, and four assistants, all of whom have had either library training or experience in editorial or secretarial work. All procedures are in accordance with the accepted principles of library economy and office practice as adapted and applied to a highly specialized institution. Contributions are made to the publications of the laboratory in other ways than by editorial and reference service; for example, by independent compilations such as the Index to publications of the Division for the period 1914–1944 (2); and in the preparation of the indexes of the annual reports and of Standard Methods. The laboratory illustrator and photographer is associated with the library department. The illustration of publications and the preparation of museum and other exhibits are facilitated by this integration of related services.

PREPARATION OF REPORTS AND PUBLICATIONS

Research

Preliminary Work.—Every member of the staff is expected to submit such proposals for special study as are indicated by advances in his particular field and within the scope of his responsibilities. When a research problem is suggested by a worker or proposed to him, the approval of the head of his department and of the director must be obtained. Application is then made to the publications section either directly, or through the secretary of his department, for information in regard to any work that may have been done in the laboratory on the subject in question. Conferences at this point with the librarian should determine also whether appropriate literature should be recommended by the library staff and, if so, to what extent, or whether other assistance is needed, such as the preparation of abstracts or translations.

Preparation and Approval of Outlines.—After the literature on the subject has been reviewed, an outline is prepared, stating (1) the problem; (2) the purpose of the investigation; (3) the conclusions to be drawn from a study of the literature; (4) the technic it is proposed to use. A copy showing the opinion of the head of the department is sent to the scientific file, recorded, and given to the director. If it receives his approval, the work may be begun. Complete original records are made on 4- by 6-inch cards or in 5- by 8-inch notebooks or otherwise as approved, and are later filed in the scientific file.

Reports.—Periodic reports of progress are made to the head of the department, read by the director, and filed in the scientific file. If the study is enlarged beyond that outlined, or if the course of investigation is changed, supplementary outlines are made; they follow the same procedure of approval as the original outline and are filed in the scientific file. All outlines and progress reports are read by the librarians so that in indexing the literature they may be informed of the trend of investigation in the laboratory.

Presentation and Publication of Papers

Preparation of Papers.—Procedures in the presentation or publication of a paper follow the same pattern as in the handling of outlines and reports. When a progress or final report on a research problem has been approved, the investigator consults with the head of his department on the advisability of presenting or publishing the results. No title is offered for presentation nor any manuscript submitted for publication without the approval of the head of the department and the director. An abstract of the material to be presented is submitted with the request for approval. It is sent by the secretary of the department in which the paper originates to the director through the publications group in the same manner as all other reports. The complete paper to be presented is usually prepared in form for publication since it is generally the custom of a society to require the deposit of manuscripts at the time of presentation for subsequent publication in its proceedings or journal. The manuscript must, therefore, be prepared sufficiently in advance of presentation to allow for such revision as may be necessary and for the preparation of lantern slides or other illustrative material. All manuscripts are reviewed and edited by the secretary of the department for form and organization. When they have been approved by the head of the department they are sent to the publications group. Here manuscripts undergo final revision. The purpose of this revision is to make the papers conform in diction and general form to the standards adopted by the laboratory and the different journals. The publications section is expected to suggest any changes necessary to achieve this end. No statement of fact may be changed or any alteration made in the substance of the paper without the approval of the author. It is, finally, sent to the director for approval and then returned to the publications section, from which, if it is to be published, it is later sent to an appropriate journal.

Scientific File.—After a paper has been made ready for presentation or publication, a copy is sent to the scientific file, together with the original records, also any microscopic preparations, specimens for the museum collection, and maps or charts.

All manuscripts are kept in the scientific file, which thus constitutes a complete, permanent record of the research of the laboratory staff. If on the completion of a study no presentation or publication is to be made, a statement to this effect is made for the record in the final report. All unpublished reports and records are confidential and may not be consulted without the permission of the director or the head of the department in which the work was done.

Publication.—Proof is read in the publications group, which is responsible for form and typographic accuracy. It is then given to the author. Reasonable changes in subject matter may be made at this time if necessary.

Reprints are ordered for binding in collected studies and for distribution to the approved laboratories of the state and in the exchange maintained with governmental departments and with other institutes and research workers.

Reports

The head of each laboratory department makes a written report of the year's activities to the director on December 1st. It is prepared for publication in the manner outlined for other publications. The annual report is published in the composite report of the Department of Health and is also issued in full as a separate publication of the Division for selective distribution.

Special reports of visitors or reports of visits made by members of the laboratory staff to other institutions are sent to the librarian to be read but are filed in the main office files.

Changes in Methods

A proposed change in standard methods is based on facts established by experience, or suggested in the literature. The results of special research are frequently applied to the improvement of a method. A suggested change is submitted to the head of the department with a statement of the reasons for the recommendation and data to support it. It is then transmitted to the Committee on Standard Methods, which is composed of representatives from each department. Upon approval by the committee, the change is referred to the director. It must be approved by the director before it is adopted.

THE LIBRARY

The library is always open to members of the staff. It is maintained for reference and reading and no materials circulate outside the laboratory.

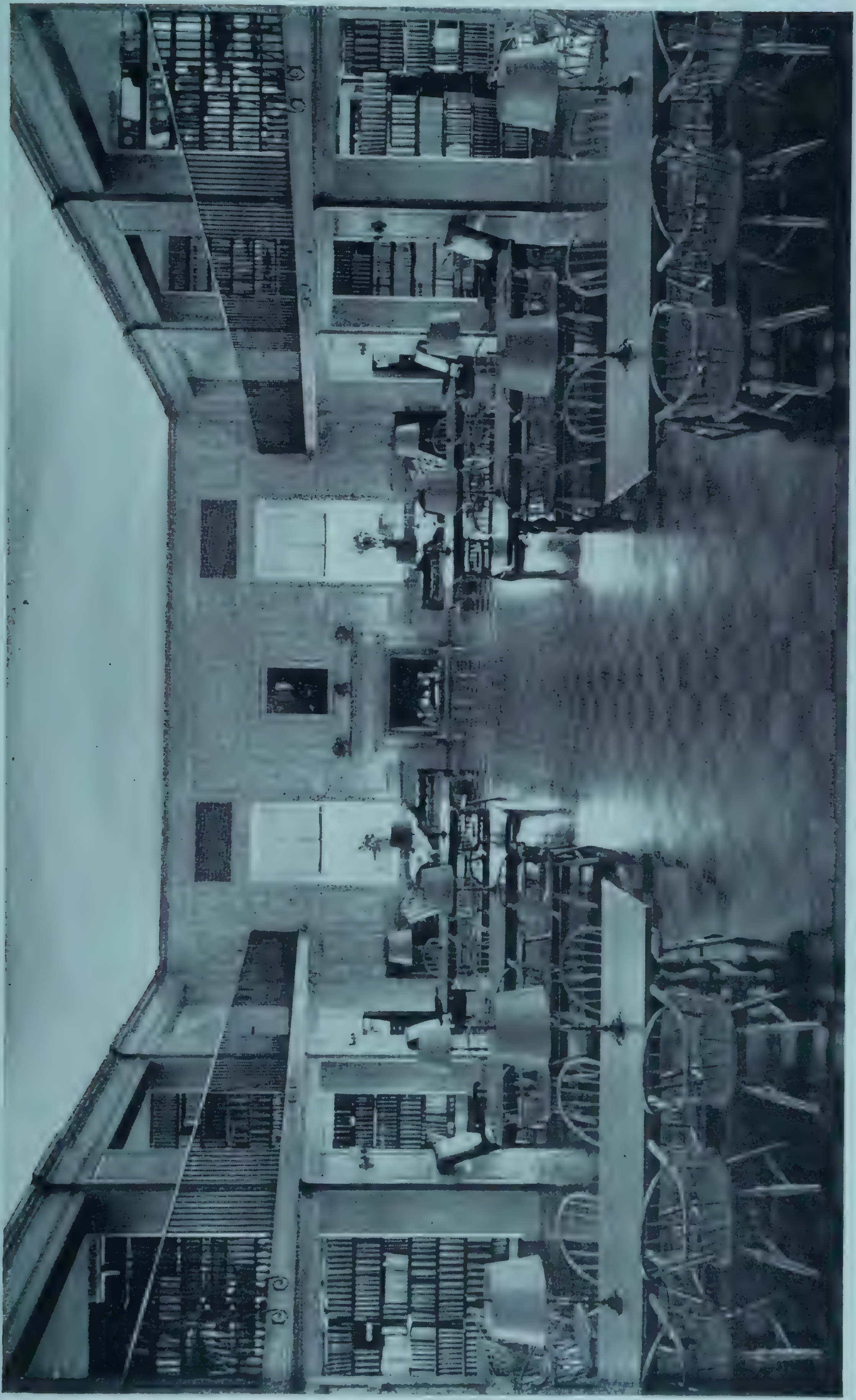


Fig. 76. The Library

The principal resource of the library is its collection of periodical literature, selectively chosen to cover the essential publications in the several different fields of the laboratory's services. A substantial collection of basic reference works and current texts is also maintained; and a large reprint file.

Catalog and Indexes

The general catalog is a dictionary card catalog. It has been developed to satisfy the needs of a highly specialized library and it is adapted to changing functions as necessity requires. Library of Congress cards are used whenever possible. In other instances the special nature of the material requires individual treatment. Serials are extensively analyzed. Subject headings are chosen from several standard lists, thus affording flexibility through comparative choice. Material represented in the catalog ranges from the simple, often valuable, pamphlet to the unusual and occasional rare item in the history of medicine.

The current literature index is a subject catalog separate from the general catalog. Flexibility in form and style is practiced in this analytical index to make it of the fullest possible service.

Methods Used in the Library

Current Literature Review.—The librarians are acquainted in the several ways outlined with the current interests and obligations of the laboratory departments, and from this point of view examine periodicals as soon as they are received and note papers of interest to members of the staff. Typewritten slips giving the complete reference are distributed to the individuals or departments concerned with the particular subject. Valuable bibliographies are thus established by individual investigators or groups. Additional carbon copies of each notice are made when the article is of interest to more than one person. One copy is filed in the library in the current literature index.

In 1943 distribution of selective lists from the index was made to laboratories approved for bacteriologic and pathologic examinations, since the critical demands during the war years called for the widest use of any service that would facilitate the work. The Council of the New York State Association of Public Health Laboratories considered the plan fully worth while and has recommended it as a permanent service (3).

Reference Questions.—Simple reference questions are given immediate attention by a member of the library staff. Requests for bibliographies, abstracts, translations, or for information about which the worker can give only inadequate data, are presented in writing. In the case of an obscure reference question, the worker is asked to state any facts that might be helpful in a search for information, and, if he himself has consulted any sources, to name them and to give the dates covered and the subjects under which he has looked. In the case of bibliographies, abstracts, and translations, the librarian confers with the worker in question and together they decide on the scope of the work and the best method to pursue.

When requests are received from any of the approved laboratories in the state, the library staff prepares a list of references and, when possible, abstracts or translates an article containing the needed information.

Instruction for New Workers.—All volunteer students come to the library on entering the laboratory (4). They are instructed in its use and are given prescribed readings during a short period of orientation. Members of the staff are also acquainted with the facilities of the department. The librarian endeavors to meet all new workers, and the staff give instruction and assistance in the arrangement and use of the resources of the library.

Interlibrary Loans.—Material not in the library is borrowed from the State Medical Library. If a book cannot be obtained there, it is borrowed from the New York Academy of Medicine, the Surgeon General's Library, or elsewhere; or photostatic or microfilm copy is procured.

MUSEUM

The museum is administered as a part of the library. Its present content has been influenced by the traditional concept of the medical museum as a collection of anatomic specimens and by the function of the laboratory in approving laboratories throughout the state for examinations in surgical pathology. Its plan of development includes, however, the demonstration of the various educational, technical, and other services of the laboratory.

The Collection of Type Cultures (see p. 87) is regarded as an important section of the museum.

All the materials in the museum files are closely integrated with the scientific file and with the library collections, and are cataloged according to the same standards.

The anatomic and pathologic material includes tissue slide units and mounted specimens. Color photographs and transparencies are replacing preserved specimens in many instances. Microscopic as well as gross material is thoroughly cataloged. The tissue collection is used for reference in routine diagnosis and for research. The approved laboratories are canvassed for items of special interest through the New York State Association of Public Health Laboratories, as are also the pathologists of the larger hospitals or specialists in certain fields. Important contributions have been received in this way and by exchanges with other museum collections. All material so received is carefully recorded as to source, with references, if published. The scope of the collection is thus not limited, although special attention has been directed to neoplastic disease to provide a reference collection for the laboratories approved for pathology and the diagnosis of cancer. Plans for the development of the museum include additions in other fields, as forensic and industrial medicine.

The major portion of the collection has come from the large reservoir of case records and pathologic specimens in the Memorial Hospital in New York. In the preparation of the units, an effort has been made to adhere to a case method of presentation. The material is designed to assist the pathologist in three directions: (1) to present the problems in diagnosis and therapy of common types of tumors, (2) to emphasize difficulties in diagnosis in borderline lesions, and (3) to afford experience in diagnosis of rare tumors. Throughout, the aim has been to afford both comprehensiveness and selectivity. The case records of the collection tend to emphasize natural historic aspects of disease in relation to diagnosis and therapeutics. Thus, in examining the cases the pathologists may detect trends toward general behavior of different types of tumors and their influence upon selection of therapy and upon prognosis (5). Additional specimens, but with less complete case histories and follow-up notes, are also available for reference or loan. They are made up of items that were assembled before the present type-collection was undertaken and of others that are added currently as they become available. The museum classification is according to anatomic system, with decimal enumeration, based on the Abbott-Johnston scheme (6).

Classification of Pathologic Specimens by Anatomic System[1]

- **00. General**
- **0. Regional**
    - 0.1 Head
        - 0.11 Scalp
    - 0.2 Face
    - 0.3 Neck
    - 0.4 Back
        - 0.41 Spine
        - 0.42 Loin
    - 0.5 Thorax
        - 0.51 Mediastinum
    - 0.6 Abdomen
        - 0.61 Abdominal wall
        - 0.62 Abdominal cavity
        - 0.63 Umbilicus
    - 0.7 Pelvis
    - 0.8 Upper extremity
        - 0.81 Scapula
        - 0.82 Axilla
        - 0.83 Shoulder
        - 0.84 Upper arm
        - 0.85 Elbow
        - 0.86 Forearm
        - 0.87 Wrist
        - 0.88 Hand
        - 0.89 Fingers (and thumb)
    - 0.9 Lower extremity
        - 0.91 Buttock
        - 0.92 Groin
        - 0.93 Hip

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[1] Specimens are classified according to system of the body, organ involved, and lesion or condition demonstrated. The main classification is determined by the system of the body from which the specimen originates; the subclassification is by organ or part of organ; designation of the lesion is given in a subheading as additional information. For example: 3.11 Digestive system Mouth Lips Carcinoma Squamous-cell.

- 0.94 Thigh
- 0.95 Knee
- 0.96 Leg
- 0.97 Ankle
- 0.98 Foot
- 0.99 Toes

1. Circulatory system
- 1.1 Pericardium
- 1.2 Myocardium
- 1.3 Endocardium
- 1.4 Heart (as a whole)
  - 1.41 Coronary arteries
- 1.5 Arteries (as a whole)
  - 1.51 Aorta and branches
  - 1.52 Pulmonary and branches
  - 1.53 Ductus arteriosus
- 1.6 Veins
- 1.7 Blood vessels

2. Respiratory system
- 2.1 Nose
- 2.2 Accessory sinuses
- 2.3 Larynx
- 2.4 Trachea
- 2.5 Bronchi
- 2.6 Lung
- 2.7 Pleura

3. Digestive system
- 3.1 Mouth (and oral cavity)
  - 3.11 Lips
  - 3.12 Tongue
  - 3.13 Teeth
  - 3.14 Gums
  - 3.15 Palate
  - 3.16 Uvula
  - 3.17 Fauces
  - 3.18 Tonsil
- 3.2 Salivary glands
  - 3.21 Parotid
  - 3.22 Sublingual
  - 3.23 Submaxillary
- 3.3 Pharynx
- 3.35 Esophagus
- 3.4 Stomach
  - 3.41 Stomach contents
- 3.5 Intestine
  - 3.51 Small intestine
  - 3.52 Appendix
  - 3.53 Large intestine
  - 3.54 Rectum and anus
  - 3.55 Intestinal contents
- 3.6 Peritoneum
- 3.65 Mesentery
- 3.66 Omentum
- 3.7 Liver
- 3.8 Gall bladder and bile ducts
  - 3.81 Bile ducts
  - 3.82 Bile
  - 3.83 Gall stones
- 3.9 Pancreas

4. Hematopoietic system
- 4.1 Bone marrow
  - 4.11 Blood
- 4.2 Lymph nodes
  - 4.21 Capsule
  - 4.22 Lymphatics
  - 4.23 Lymph
- 4.3 Spleen

4.5. Endocrine system
- 4.51 Thymus
- 4.52 Suprarenal gland
- 4.53 Carotid gland
- 4.54 Pituitary gland
- 4.55 Pineal gland
- 4.56 Thyroid
- 4.57 Parathyroid glands
- 4.58 Insular tissue of pancreas

5. Genitourinary system
- 5.1 Kidney
  - 5.11 (Renal) Pelvis
- 5.2 Ureter
- 5.3 Bladder
  - 5.31 Urine
- 5.4 Urethra
- 5.5 Prostate
- 5.6 Seminal vesicles
- 5.61 Vas deferens
- 5.62 Spermatic cord
- 5.7 Testis
- 5.71 Epididymis
- 5.72 Tunica vaginalis
- 5.75 Scrotum
- 5.8 Penis
- 5.9 Perineum

6. Female genitalia
- 6.1 External (genitalia)

  - 6.11 Vulva
  - 6.12 Perineum
- 6.2 Vagina
- 6.3 Uterus
  - 6.35 Cervix
- 6.4 Fallopian tube
- 6.5 Ovary (and parovarium)
- 6.6 Pelvic peritoneum
- 6.7 Breast[2]
- 6.8 Generative system in pregnancy
  - 6.81 Vulva
  - 6.82 Vagina
  - 6.83 Uterus
  - 6.84 Fallopian tube
  - 6.85 Ovary (and parovarium)
  - 6.86 Pelvic peritoneum
  - 6.87 Breast
- 6.9 Ovum as a whole
  - 6.91 Membranes
  - 6.92 Placenta
  - 6.93 Fetus

7. Nervous system

- 7.01 Brain (as a whole)
- 7.1 Membranes
  - 7.11 Dura
  - 7.12 Pia arachnoid
  - 7.13 Cerebrospinal fluid
- 7.2 Cerebral vascular system
  - 7.21 Arteries
  - 7.22 Veins
  - 7.23 Venous sinuses
  - 7.24 Choroid plexus
- 7.3 Cerebrum
  - 7.31 Cerebral hemisphere (cortex, corpus striatum, ventricles, olfactory tract)
  - 7.32 Thalamus, hypothalamus, and ventricles
  - 7.33 Midbrain (corpora quadrigemina, cerebral peduncles, aqueduct of Sylvius, and perforated space)
- 7.4 Cerebellum, pons, and medulla
  - 7.41 Cerebellum
  - 7.42 Pons
  - 7.43 Medulla
  - 7.44 Fourth ventricle
- 7.5 Spinal cord
- 7.6 Spinal nerves and plexuses
- 7.7 Sympathetic system
- 7.8 Eye
  - 7.81 Orbit
  - 7.82 Lids
  - 7.83 Lachrymal apparatus
  - 7.84 Conjunctiva
  - 7.85 Cornea and sclerotic
  - 7.86 Refractive system
    - 7.861 Anterior chamber
    - 7.862 Lens
    - 7.863 Vitreous
  - 7.87 Vascular coat
    - 7.871 Iris
    - 7.872 Ciliary
    - 7.873 Choroid
  - 7.88 Retina
  - 7.89 Optic nerve
- 7.9 Ear
  - 7.91 External ear (auricle and meatus)
  - 7.92 Middle ear (membrane, ossicles, mastoid, Eustachian canal)
  - 7.93 Internal ear (labyrinth, semi-circular canals, cochlea, organ of Corti)

8. Musculocutaneous system

- 8.01 Fat[3]
- 8.1 Skin[4]
  - 8.11 Sweat glands
  - 8.12 Sebaceous glands
- 8.2 Hair
- 8.3 Nails
- 8.4 Muscles
- 8.5 Fascia
  - 8.51 Superficial (fascia)
  - 8.52 Deep (fascia)
- 8.7 Tendons
  - 8.71 Tendon sheaths
- 8.8 Ligaments
- 8.9 Bursae

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[2] Lesions of the male breast are classified under 8.1.

[3] Fatty tumors of a specific organ are classified under the system of that organ, for example, 3.7 Digestive system—Liver—Liposarcoma; all others under 8.01.

[4] Includes male breast; female breast, 6.7.

- **9. Osseous and articular system**
  - 9.1 (Bones of) Cranium
  - 9.2 (Bones of) Face
  - 9.3 Vertebral column
  - 9.4 Sternum
  - 9.5 Ribs
  - 9.6 Upper extremity
    - 9.61 Scapula
    - 9.62 Clavicle
    - 9.63 Humerus
    - 9.64 Radius
    - 9.65 Ulna
    - 9.66 (Bones of) Hand
      - 9.661 Carpus
      - 9.662 Metacarpus
    - 9.67 (Bones of) Fingers
  - 9.7 Pelvis girdle
  - 9.8 Lower extremity
    - 9.81 Os innominatum
    - 9.82 Femur
    - 9.83 Patella
    - 9.84 Tibia
    - 9.85 Fibula
    - 9.86 (Bones of) Foot
    - 9.87 (Bones of) Toe
  - 9.9 Joints
    - 9.91 Epiphysis
    - 9.92 Synovial membrane
    - 9.93 Synovial fluid

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APPENDIX

APPENDIX A

PROVISIONS OF NEW YORK STATE LAWS AND SANITARY CODE RELATING TO APPROVED LABORATORIES

PUBLIC HEALTH LAW

ARTICLE II

Section 4-b. Duties of commissioner with respect to laboratories.—The commissioner of health shall establish and maintain one or more laboratories with such expert assistants and such facilities as are necessary for routine examinations and analyses, and for original investigations and research in matters affecting public health. He shall have authority to make, at the expense of the state, such examinations and analyses at the request of any health officer or of any physician. He may enter into contracts with laboratories in localities accessible to the various portions of the state for the prompt examination of specimens received from local health officers or physicians and for the immediate report, thereon, at the expense of the state; provided that all such laboratories shall conform to the standards of efficiency established by the public health council, and that no obligation shall be incurred by the commissioner in excess of the sums available therefor.

The commissioner of health may issue to laboratories certificates of approval covering such laboratory examinations as the state sanitary code may require to be made in laboratories approved for such examinations, and may prescribe the conditions under which such approvals will be granted. Notwithstanding any other conditions which he may prescribe, such an approval shall not be issued hereafter to a laboratory, not heretofore approved, unless the director, bacteriologist or pathologist in charge of such examinations shall possess such educational and technical qualifications as the public health council shall establish.

ARTICLE II-A

Section 18-d. Serological blood test for syphilis of pregnant women.—Every physician attending pregnant women in the state during gestation shall in the case of every woman so attended take or cause to be taken a sample of blood of such woman at the time of first examination, and submit such sample to an approved laboratory for a standard serological test for syphilis. . . . The term "approved laboratory" means a laboratory approved for this purpose by the state department of health, or in the city of New York by the department of health of such city. . . .

ARTICLE III

[*State Aid for Laboratory Service*]

Section 20-c. Laboratories.[1]—The board of supervisors of any county may establish therein a laboratory or laboratories which shall serve the whole or part of the county. In the resolution of the board of supervisors establishing such laboratory they shall define the area which it is intended to serve, which area from time to time may by resolution be altered. Provided, however, that in defining such area the territory included in a town shall not be divided and provided further, that no city or any part thereof shall be included in the area so defined, unless the mayor and the common council, or the officials exercising similar powers, shall have consented thereto. The services of such laboratories shall be rendered at a moderate charge or free.

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[1] See also Section 20-b, subd. 11, which follows Section 20-h.

If, however, it is the intention of the board of supervisors to apply for state aid for such proposed laboratory, under the provisions of section twenty-h of this article, an application for such state aid for such proposed laboratory shall be transmitted by the board of supervisors to the state commissioner of health. On or before January first of each year the state commissioner of health shall file with the governor a statement of the aggregate amount of such applications for state aid for proposed new laboratories in addition to the aggregate amount of applications for state aid for laboratories already established.

Such sums, as revised in the discretion of the governor, shall be the estimate of state aid under this article to be used in the preparation of the executive budget.

Subsequent to enactment of the appropriation by the legislature the state commissioner of health shall revise such applications for state aid as necessary to bring the aggregate of all applications within the total appropriations available.

The board of supervisors may, in lieu of the establishment of a laboratory and with the approval of the state commissioner of health, provide for laboratory service by contracting with an established laboratory which is conveniently located and when such service shall have been provided, shall be entitled to such state aid as would be provided for under this act if the amount expended for service were expended for maintenance and operation of a laboratory established in accordance with the provisions of this act.

Upon the petition signed by two hundred or more taxpayers of the county or district to be served by such a laboratory, the governing body of that county or district at the next date for filling an elective office shall hold a referendum upon the question of establishing a laboratory the construction of which is to be financed by taxes levied for the fiscal year in which the expenditures therefor are to be made. If a majority of the votes cast are in favor of establishing such a laboratory it shall be mandatory upon the governing body of that county or district to take the steps necessary for the establishment and maintenance of such a laboratory as provided for by this act. Nothing contained in this section shall be construed to prevent the financing of any expenditure, in whole or in part, pursuant to the local finance law. (Added by L. 1923, ch. 638; amended by L. 1940, ch. 731, by L. 1943, ch. 710, by L. 1944, ch. 606, and by L. 1945, ch. 338, effective September 2, 1945.)

Section 20-d. Powers of boards of supervisors in relation to laboratories.—The board of supervisors, when they shall have determined to establish such a laboratory shall have the following powers:

1. To acquire by purchase, exchange or otherwise, necessary real property, building or rooms or to erect necessary buildings.

2. To cause to be assessed, levied and collected in the same manner as other charges against the county, such sums of money as it shall deem necessary for laboratory purposes. Provided, however, that where a laboratory is intended to serve less than a whole county the expenditures made in connection therewith shall be assessed only against the area served by the laboratory.

3. To accept and hold in trust for the county any grant or devise of land or any gift or bequest of money or other personal property or any donation to be applied, principal or income, or both, for the benefit of said laboratory.

4. To appoint a board of managers for the laboratory which shall consist of at

least five members, two of whom shall be physicians duly licensed to practice in the state of New York. The county medical society may present to the board of supervisors a list of physicians residing in the county from which the board may choose the medical members of the board of managers. The board of managers shall hold a meeting at least four times in each year, and at such other times as it may deem necessary and each member attending meetings shall receive his actual and necessary expenses incident thereto, to be audited and paid in the same manner as other expenses of the laboratory. In counties having a county board of health and a county health officer, the president of the county board of health and the county health officer shall be members of the board of managers ex-officio. The members of such board, with the exception of the members ex-officio, shall first be appointed so that the term of one member shall expire within one year from the first day of January of the year in which he shall have been appointed, the term of another member shall expire within two years of the first day of January of the year in which he shall have been appointed, the term of another member shall expire within three years of the first day of January of the year in which he shall have been appointed, the term of another member shall expire within four years of the first day of January of the year in which he shall have been appointed, and the term of another member shall expire within five years of the first day of January of the year in which he shall have been appointed. Thereafter the terms of membership shall be made for five years from the first day of January of the year in which the appointment is made. Nothing in this act shall be construed to repeal or amend any provision of law not inconsistent herewith relating to laboratories in counties or to abrogate any powers of boards of supervisors relating thereto. (Added by L. 1923, ch. 638; amended by L. 1943, ch. 710, by L. 1944, ch. 606, and by L. 1945, ch. 338, effective September 2, 1945.)

Section 20-e. Powers of boards of managers of laboratories.—The board of managers of each laboratory shall have the following powers and duties:

1. To elect a chairman, vice-chairman and a secretary.

2. To appoint a director or a bacteriologist in charge of the laboratory. Any person appointed as such director shall comply with such qualifications as may be prescribed by the public health council.

3. To fix the salaries of the director of the laboratory and all other employees within the limits of the appropriation made therefor by the board of supervisors.

4. To exercise general management and control of said laboratory, of the grounds, buildings, rooms, employees and of all matters relating to the government, discipline, contracts and fiscal concerns thereof.

5. To make such rules and regulations as may be necessary in relation to the administration of the laboratory and the fees to be charged for laboratory service, not inconsistent with the provisions of this act.

6. Notwithstanding any other general or special law, to erect all additional buildings found necessary after the laboratory has been placed in operation and make all necessary improvements and repairs within the limits of the appropriations made therefor.

7. To make to the board of supervisors annually at such time as said supervisors shall direct a detailed report of the operation of the laboratory during the calendar year, the number and kind of specimens examined and the results of such examinations, together with suitable recommendations and such other matters as may be required of them and full and detailed estimates of the appropriations required during the ensuing year for all purposes including maintenance, erection of buildings, repairs, renewals, extensions, improvements, betterments or other necessary purposes.

8. To establish branch laboratories if the area to be served by the laboratory is so

large, if its topography is such as to make access to the laboratory difficult, or for any other reason such action seems reasonable or desirable. (Added by L. 1923, ch. 638.)

Section 20-f. Powers of the director of a laboratory.—The director or the bacteriologist in charge of such laboratory, subject to the board of managers, shall:

1. Equip the laboratory with all necessary furniture, appliances, fixtures and other needed facilities for the conduct of laboratory work and purchase all necessary supplies within the appropriations made therefor.

2. Have general supervision and control of the internal affairs and work of the laboratory. He may make and enforce such rules, regulations and orders as he may deem necessary, not inconsistent with law or with the rules and regulations of the board of managers.

3. Appoint employees of the laboratory within the limits of his appropriations, and remove them.

4. Cause to be kept proper accounts and records of the business and operation of the laboratory, including such records relating to specimens examined and render such reports as may be required by law or by the regulations of the state commissioner of health; certify all bills and accounts including salaries and wages, and transmit them to the board of supervisors which shall provide for their payment in the same manner as other charges against the county. The board of supervisors of a county not having a purchasing agent may make an appropriation for the maintenance of such laboratory and direct the county treasurer to pay all bills, accounts, salaries and wages which are approved by the director of the laboratory within the amount of such appropriation, subject to such regulations as to the payment and audit thereof as the board of supervisors may deem proper. (Added by L. 1923, ch. 638.)

Section 20-g. Laboratories in cities.—Nothing contained in section twenty-e to twenty-f hereof inclusive, shall be construed to repeal or amend any provision of law under which any health function or activity may be carried on in any city, or to transfer or affect any authority in relation to health activities now being exercised in any city by any public board or officers. Provided, however, that any public board or officers of a city now exercising health functions may, with the approval of the mayor, contract with the board of managers of any laboratory for the purpose of cooperation and to join and share facilities. If a city desires to avail itself of the state aid provided for in section twenty-h of this act, the common council or any body exercising similar powers in any city with the approval of the board of estimate, if such exists, shall have the power to establish a laboratory in such city. Upon the establishment of such laboratory by such city, all the powers and duties of the board of supervisors, in relation to laboratories hereinbefore provided for, shall devolve upon the common council or other body exercising similar powers, except that the mayor shall appoint the board of managers of such laboratory and that the salaries of the director and other employees of the laboratory, and contracts to be made for, by, or on behalf of the laboratory and appropriations for the acquisition of sites and buildings and for maintenance, shall all be under the control of the same officials as now have control of similar items and shall be governed by the same provisions of law. In cities when more than one laboratory is established, but one board of managers shall be appointed, which shall have jurisdiction over all the laboratories established or operating under this act in said city. In cities of the second and third class the president of the local board of health, if such office exists, and the local commissioner of health or health officers shall be ex-officio members of the board of managers of the laboratory. (Added by L. 1923, ch. 638.)

Section 20-h. State aid.—Where a laboratory as hereinbefore provided for shall have been established, the state, through the legislature, shall provide the following aid; a grant of an amount not to exceed one-half of the actual cost of maintenance of the laboratory or laboratories not in excess of seven thousand five hundred dollars per annum for each laboratory and of twenty-five hundred dollars toward the initial installation and equipment of such laboratory.

The salaries and traveling expenses of employees of the state department of health engaged in supervision or inspecting laboratories unless otherwise provided for and other expenses necessarily incurred by the state department of health in the execution and enforcement of this act shall be paid from the sum appropriated toward maintenance and operation of laboratories as hereinbefore specified.

The board of supervisors of any county or the common council or other body exercising similar powers of any city which has heretofore established and is maintaining a laboratory or which is already providing laboratory service by contract with another laboratory may apply for state aid in maintaining and operating such laboratory or in otherwise providing such laboratory service and upon complying with the provisions and requirements of this act, shall be entitled to such state aid, provided however, that in the event that the total amount appropriated for state aid is not sufficient to pay in full the state aid and other expenses hereinbefore provided for, counties or cities establishing laboratories or providing laboratory service under this act shall be given precedence and any balance remaining of moneys appropriated under this act, after the payment of full state aid to such counties or cities, shall be allotted to such counties or cities as have heretofore established and are now maintaining laboratories or are providing laboratory service by contract and have applied for state aid.

The work of all laboratories, except in the city of New York, established or receiving aid in accordance with the provisions of this act shall be inspected and standardized by the state department of health, and no state aid shall be given to any laboratory under the provisions unless the area of the district. site, design and construction of the buildings, equipment, work and conduct of such laboratory shall be first approved in writing, after inspection, by the state commissioner of health or his representative.

The comptroller after receiving the written approval of the state commissioner of health, hereinbefore provided for, shall determine the amount due in any one year to the various laboratories in the state under this act, and shall draw his warrant upon the state treasurer in favor of the county treasurer of each county or the city treasurer of each city for the total amount to be paid to each laboratory in such county or city or in consideration of laboratory service contracted for in such county or city as shall be determined by him and shall indicate the amount to be paid to each laboratory or to each county or city in consideration of laboratory service contracted for. The county or city treasurer shall pay out such amount in the same manner and upon the same vouchers and proof as all other moneys devoted to such laboratory.

The board of managers of a laboratory may appeal from any decision of the comptroller or of the state commissioner of health or any refusal to furnish the written approval herein provided for to the governor, and the action of the governor shall be final and conclusive and his approval, if granted, shall be accepted by the comptroller in lieu of the certificate of the commissioner of health herein provided for.

Laboratories established or receiving aid under this section, except in the city of New York, shall be under the supervision of the director of the division of laboratories and research. (Added by L. 1923, ch. 638; and amended by L. 1941, ch. 634, by L. 1944, ch. 298, and by L. 1945, ch. 37.)

ARTICLE III

Section 20-b. County health districts. . . . 11.—The board of supervisors of any county in which a county health district has been established, the boundaries of which are coterminous with the county, shall have power to abolish the board of managers of the county laboratory of such county, established and operated under the provisions of sections twenty-e to twenty-f, both inclusive, of the public health law, and to confer the powers and duties of such board of managers of such county laboratory upon the county board of health. The board of supervisors of any such county, with the approval of the state commissioner of health, may abolish the board of managers of the county tuberculosis hospital established and operated in such county under the provisions of sections forty-five to forty-nine-e, both inclusive, of the county law, and confer the powers and duties of such board of managers upon the county board of health. . . .

ARTICLE III

Section 25. (Reporting of infectious and contagious or communicable diseases.) . . . —Whenever an examination for diagnosis by a laboratory or by any person other than the physician in charge of the person from whom the specimen is taken, of any specimen, discloses the existence of a case of communicable disease, or the results obtained in the examination of a specimen are needed for purposes of release from quarantine or observation, the person in charge of such laboratory or the person making such examination shall immediately report the same, together with all the facts in connection therewith, to the local or state health official to whom the attending physician is required to report such case. The person in charge of such laboratory or the person making such examination shall keep for a period of time to be specified by the state commissioner of health a record of all the facts in connection with such examination, including the identity of the person from whom the specimen is taken and the name of the physician, if any, sending such specimen. . . .

Section 25-b. Reporting of cancer and other malignant tumors. . . .—Whenever an examination of a tissue specimen in a laboratory discloses the existence of cancer or other malignant tumor, the person in charge of such laboratory or the person making such examination shall immediately report the same together with all the facts in connection therewith to the local or state health official to whom the attending physician is required to report such case. . . .

DOMESTIC RELATIONS LAW

ARTICLE III

Section 13-a. Physician's examination and serological test of applicant for marriage license.—1. Except as herein otherwise provided, no application for a marriage license shall be accepted by the town or city clerk unless accompanied by or unless there shall have been filed with him a statement or statements signed by a duly licensed physician . . . that each applicant has been given such examination, including a standard serological test, as may be necessary for the discovery of syphilis. . . .

3. Each physician's statement shall include the name and address of the applicant, a statement that a serological test for syphilis was performed, the date upon which the specimen was taken and the name and location of the laboratory in which such test was made. The result of the serological test shall not be included in such statement. . . . Upon a separate form a detailed report of the laboratory test, showing the result of the test, shall be transmitted by the laboratory to the physician, and a copy of this report when positive shall be submitted to the district state health officer, or, in a city of over fifty thousand population, or in a county health district, to the department of health of such city, or county as the case may be, . . .

4. A standard serological test shall be a laboratory test for syphilis approved by the state commissioner of health and shall be performed by the state department of

health, or in the city of New York by the department of health of such city, or at a laboratory approved for this purpose by the state department of health or, in the city of New York, by the department of health of such city. . . .

SANITARY CODE[2]

CHAPTER II

Regulation 2a. Reporting cases of communicable disease diagnosed after death.—If a pathologist, coroner, medical examiner, or other person determines from examination of a corpse or from history of the events leading to death that at the time of death this individual apparently was affected with a communicable disease, he shall report the case promptly to the proper health authority according to the manner indicated in regulation 2 of this chapter as if the diagnosis had been established prior to death.

Regulation 9. Physician to submit specimens for laboratory examination in cases or suspected cases of certain communicable diseases.—A physician in attendance on a person affected with or suspected of being affected with any of the diseases mentioned in this regulation shall submit to an approved laboratory, or to the laboratory of the state department of health for examination such specimens as may be designated by the state commissioner of health, together with data concerning the history and clinical manifestations pertinent to the examination: anthrax, chancroid, Asiatic cholera, diphtheria, bacillary dysentery, glanders, malaria, meningococcal meningitis or meningococcemia (septicemia), ophthalmia neonatorum, paratyphoid fever, plague, streptococcal sore throat (including scarlet fever), syphilis, tularemia, typhoid fever, typhus fever, undulant fever, and Vincent's angina.

Directions Governing Submission of Specimens

The following specimens should be submitted for laboratory examination, together with pertinent data concerning the history and clinical manifestations necessary for the examination.

Anthrax

1. Exudate from the lesion on a sterile swab (tube outfit with swab);
2. Films of the exudate on glass slides (slide outfit).

Chancroid

In order to detect concurrent syphilitic infection, submit the specimens specified under syphilis.

Cholera, Asiatic

1. A specimen of feces in a sterile container without preservative (jar outfit);
2. Ten milliliters of blood to be examined for evidence of typhoid fever (typhoid tube outfit).

Diphtheria

A culture from the throat on Loeffler's blood-serum medium and, if symptoms of rhinitis are observed, a culture from the nose also (diphtheria culture outfit).

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[2] The Sanitary Code of New York State is effective in all sections of the state except in the City of New York.

Dysentery, Bacillary

1. A specimen of feces (typhoid jar outfit containing buffered 30-per-cent glycerol);
2. Ten milliliters of blood to be examined for evidence of typhoid fever (typhoid tube outfit).

Glanders

1. Ten milliliters of blood (typhoid tube outfit);
2. A specimen of discharge on a sterile swab (tube outfit with swab);
3. Films of discharge on glass slides (slide outfit).

Malaria

Both thick and thin films of blood on glass slides collected preferably between twelve and twenty-four hours after a chill (slide outfit).

Meningococcal Meningitis

A specimen of spinal fluid in a sterile container (tube outfit—swab or needle removed).

Meningococcemia (septicemia)

Blood cultural tests should, if possible, be made in a nearby approved laboratory. Otherwise 10 ml. of blood shall be submitted (typhoid tube outfit).

Ophthalmia Neonatorum

Films of the exudate from the eye on glass slides (gonorrhea slide outfit).

Plague

1. A specimen of discharge or aspirated fluid, if a bubo is present (tube outfit with swab);
2. Ten milliliters of blood (typhoid tube outfit);
3. In the pneumonic type of plague, a specimen of sputum (jar outfit).

Streptococcal Sore Throat (including Scarlet Fever)

A culture from the throat on Loeffler's blood-serum medium, and the swab used in making the culture (diphtheria culture outfit).

Syphilis

1. Fluid from the lesion to be examined for Treponema pallidum (chancre fluid outfit containing capillary tubes);
2. Ten milliliters of blood for the complement-fixation (Wassermann) test (syphilis tube outfit);
3. When laboratory tests fail to disclose evidence of syphilitic infection, 10 ml. of blood for the complement-fixation (Wassermann) test, taken at weekly intervals until eight weeks have elapsed following the appearance of the primary lesion, unless evidence of syphilis is obtained earlier.

Tularemia

1. Ten milliliters of blood (typhoid tube outfit);
2. If ulcerating lesions are present, films of discharge on glass slides (slide outfit), and a specimen of discharge on a sterile swab (tube outfit with swab).

Typhoid and Paratyphoid Fever

1. Ten milliliters of blood (typhoid tube outfit);
2. A specimen of fluid feces (typhoid jar outfit containing buffered 30-per-cent glycerol and, if there is evidence of localization in the genitourinary tract, a specimen of urine (typhoid jar outfit containing buffered 30-per-cent glycerol).

Typhus Fever

1. Ten milliliters of blood (typhoid tube outfit);
2. A specimen of feces to be examined for evidence of typhoid fever (typhoid jar outfit containing buffered 30-per-cent glycerol).

Undulant Fever

Ten milliliters of blood (typhoid tube outfit).

Vincent's Angina

1. Films of the exudate on glass slides (slide outfit);
2. A culture from the exudate on Loeffler's blood-serum medium, to be examined for diphtheria bacilli and hemolytic streptococci (diphtheria culture outfit).

Regulation 10. Health officer to confine animal which has bitten person; to kill suspected rabid animal and send head of such to laboratory.[3]—Whenever . . . the health officer is notified of an animal which is of a species subject to rabies, and which has bitten any person, he shall cause the animal to be confined for one week unless such animal develops active symptoms of rabies within that time in which case it shall be killed under the direction of the health officer. . . .

Whenever any animal that has or is suspected of having rabies dies or is killed it shall be the duty of the health officer to cause the head of such animal to be removed and sent immediately, properly packed, with a complete history of the case to a laboratory approved for this purpose by the state commissioner of health.

CHAPTER III

Regulation 1. [Bio-assays of vitamin D milk to be made in approved laboratories.] . . . the term "Vitamin D milk" means milk in which the Vitamin D content has been artifically increased to not less than 135 international units per quart as determined by semi-annual bio-assays made in a laboratory approved for the purpose by the state commissioner of health. . . .

Regulation 5. [Bacterial milk counts to be made in approved laboratories.] . . . Bacterial counts to be used for the purpose of grading shall be made by the standard agar plate method upon not less than four samples of milk or cream, each sample to be taken on a different day. Whenever more than one of four successive counts are higher than the limits herein prescribed for the grade, the milk and/or cream shall be considered not to meet grade requirements. Such bacterial counts, to be so used, shall be made only in a laboratory approved for such purpose by the state commissioner of health. Thereafter, at intervals of not more than three months, official samples of milk and cream before pasteurization and as delivered to consumers shall be taken and bacterial counts made. . . .

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[3] The specimen should be kept cold. Placing the head in a pail containing cracked ice is a satisfactory method. If messenger service is not available, the head should be shipped in ice and packed with sufficient sawdust to absorb the water as the ice melts. The outside container should be as nearly water-tight as possible. Outfits are furnished the district state health officers for the submission of specimens to the Division of Laboratories and Research, Albany and New York City. (See "Appendix C," p. 921.)

CHAPTER IV

Regulation 6. Inspection of laboratories.—The state commissioner of health, or his authorized representative, shall have authority to inspect every bacteriological, chemical or pathological laboratory doing work for the health authorities of the state or of any county or municipality therein or making any examinations which the sanitary code may require to be performed in an approved laboratory. He may advise the person in charge of such laboratory as to the methods or procedures relative to such examinations, and he may report the result of the inspection to the authorities of the county or municipality in which the laboratory is located.

Regulation 7. Tissue removed at operation or necropsy to be examined in certain cases.—Representative specimens, or sections for microscopic examination, of tissue removed at operation or at necropsy which require laboratory examination as an aid in the diagnosis, prevention, or treatment of disease or to determine the cause of death shall be submitted to an approved laboratory, to the division of laboratories and research, Albany or New York city or to the state institute for the study of malignant diseases, Buffalo.

CHAPTER V

Regulation 3. Protection, supervision, maintenance of records of operation and sampling of public water supplies....(d) Samples of water, except those collected for examination by the state department of health, or those collected for examination at a water treatment plant for the control of the operation of such plant, shall be collected from a tap or taps on the distribution system by the local health officer or by the person in charge of any public water supply at as frequent intervals as may seem necessary to them to insure adequate control of the sanitary quality of the supply and such samples shall be submitted for examination to a laboratory approved for the purpose by the state commissioner of health. Samples of water submitted to such laboratories shall be accompanied by all pertinent data relative to the supply. . . .

CHAPTER XI

[*Qualifications of Persons in Charge of Approved Laboratories*]

Regulation 18. Definitions.—The terms "director," "pathologist," and "bacteriologist," as used in this section, shall be construed to mean respectively, directors of laboratories, directors or their assistants in charge of pathological examinations in laboratories, and bacteriologists in charge of laboratories, or in charge of bacteriological examinations in laboratories, in instances where such laboratories have been approved pursuant to section 4-b of the public health law.

Regulation 19. Qualifications required.—No person shall hereafter be appointed as a director, pathologist, or bacteriologist, unless he shall possess at the time of appointment the qualifications hereinafter prescribed for such position.

Regulation 20. Preliminary qualifications.—All directors, pathologists and bacteriologists shall possess the integrity and ability to conduct a laboratory in which satisfactory standards of work can be maintained; directors and pathologists shall be graduates in medicine of schools recognized by the regents of the university of the state of New York and licensed to practice medicine or eligible for examination for license to practice medicine in the state of New York; and bacteriologists shall possess the educational requirements for a doctorate degree in science, public health or medicine as prescribed by a university holding membership in the association of American universities: Provided, that under special conditions any or all of the qualifications relating to education or experience may be waived by the public health council.

Regulation 21. Qualifications, directors.—The qualifications for directors shall include an adequate knowledge of pathology and bacteriology and, subsequent to graduation, at least four years' training and experience in pathological and bacteriological work approved by the public health council: Provided, that under special conditions any or all of the qualifications relating to education or experience may be waived by the public health council.

Regulation 22. Qualifications, pathologists.—The qualifications for pathologists shall include an adequate knowledge of pathology and, subsequent to graduation, at least four years' training and experience in pathological work, approved by the public health council, of which at least one year shall have been devoted to training and experience in the diagnosis of neoplastic disease: Provided, that under special conditions any or all of the qualifications relating to education or experience may be waived by the public health council.

Regulation 23. Qualifications, bacteriologists.—The qualifications for bacteriologists shall include, subsequent to graduation, at least four years' experience or training in pathology and bacteriology or in bacteriology alone, approved by the public health council: Provided, that under special conditions any or all of the qualifications relating to education or experience may be waived by the public health council.

At a meeting on January 15, 1937, the public health council adopted the following resolution relating to the approval of laboratories: "Resolved, that, diagnostic laboratory service being intimately concerned with the practice of medicine, the state commissioner of health be advised that a laboratory offering diagnostic service should not be approved unless, in addition to meeting other conditions which may be prescribed, the person actively in charge is licensed to practice medicine or eligible for examination for license to practice medicine in the state of New York."

CHAPTER XIV

Regulation 3. Cleansing and disinfection of eating, drinking and cooking utensils.—All eating, drinking and cooking utensils shall be so cleansed and disinfected as to be free from bacilli of the coliform group and to have a total bacterial count of not more than 100 per utensil as determined by test in a laboratory approved for the purpose by the state commissioner of health.

PROCEDURE FOR APPROVAL OF LABORATORIES

Since approval is voluntary, the director or person in charge of a laboratory must first request approval by filing application specifying his qualifications. If these qualifications meet the requirements of Chapter XI of the Sanitary Code and the physical equipment of the laboratory is approved, the applicant demonstrates his ability in comparative examinations. Agreements to conduct the work in an ethical manner and to maintain the technical standards required are a part of the conditions of approval.

Certificates of approval are issued annually. They are valid until the 31st day of December of the year in which issued unless sooner revoked. Approval terminates automatically with any change in the responsibility for the direction of the work. New appointees must qualify in the usual manner.

The New York State Sanitary Code does not apply to the City of New York. Laboratories there whose directors wish to make official examinations of specimens from patients living outside of the city or to act as consultants in districts where the State Sanitary Code is effective are approved on the same basis as laboratories located elsewhere in the state.

Four years of post-graduate training and experience in the department of pathology of a medical school recognized by the Regents of the University of the State of New York, including training and experience in pathology, bacteriology, and related departments, or an equivalent combination of training and experience have been considered as meeting the requirements for directors and pathologists outlined in Regulations 21 and 22 of the Sanitary Code.

Directors, pathologists, and bacteriologists must either be on full time or devote the major part of their time and attention to the work of the laboratory. When they cease active laboratory work for a long period of years, their qualifications must be reviewed in the light of advances in knowledge in bacteriology and pathology that have taken place in the interim, before approval is issued.

Persons in charge of laboratories approved for bacteriologic examinations of milk and water and for examinations concerned with eating, drinking, and cooking utensils must have had adequate training and experience in the technical procedures involved and, in addition, must be thoroughly versed in the principles of sanitary science in order that they may be competent to interpret the significance of the laboratory findings. Those in charge of sanitary chemical analyses of water in laboratories approved for this work must also have had ample training in chemistry, including courses of instruction in analytical chemistry. Persons in charge of laboratories approved for bio-assays of vitamin D milk must have had adequate training and experience in the technical procedures and must be competent to interpret the significance of the laboratory findings. Laboratories in which samples of pasteurized milk and cream are examined must be approved also for the phosphatase test for detection of pasteurization.

APPENDIX B

LIVE PATHOGENIC MICROORGANISMS OR VIRUSES

PUBLIC HEALTH LAW

ARTICLE XXIII

Section 450. Handling of live pathogenic microörganisms or viruses.—No person other than a licensed practitioner of medicine, dentistry, or veterinary medicine or a person under the direct supervision of a licensed practitioner of medicine, dentistry, or veterinary medicine shall possess or cultivate live pathogenic microörganisms or viruses other than vaccine virus unless he has satisfied the state commissioner of health that such microörganisms or viruses in his possession will not become a menace to the public health and unless he shall hold a permit issued within the preceding twelve months by the state commissioner of health or in the city of New York, by the city commissioner of health, or their authorized representatives so stating. The state commissioner of health or in the city of New York, the city commissioner of health, is authorized to rescind such a permit at any time for a cause.

Section 451. Registration of places where live pathogenic microörganisms are handled.—All places where live pathogenic microörganisms or viruses other than vaccine virus are handled or cultivated, shall be registered with the state department of health, or in the city of New York with the department of health of such city, and a registration number shall be issued to each place so registered. Registration and application for this registration number shall be made by the person in charge of the place where such microörganisms or viruses are handled. The registration number shall be valid through April thirtieth of each calendar year and may be renewed upon application on May first of each year. A registration fee of one dollar shall be charged to cover the cost of issuing the registration number. This registration does not apply to laboratories maintained by the federal government, state, a municipality or a county.

Section 452. Sale or other disposal of live pathogenic microörganisms or viruses.—All live pathogenic microörganisms or viruses other than vaccine virus when given away or sold shall bear a label on the container showing the registration number of the distributor which has been issued by the state department of health, or in the city of New York by the department of health of such city, for the handling of pathogenic microörganisms or viruses, the name of the person obtaining the material, and the destination of the pathogenic microörganisms or viruses, and no person shall sell or convey any live pathogenic microörganisms or viruses other than vaccine virus to any other person without permission of the state commissioner of health, or in the city of New York without the permission of the city commissioner of health. This section does not apply to diseased tissue, exudate, or other specimens which are sent by physicians to laboratories for examination as an aid in the diagnosis or control of disease.

Section 453. Violation of article.—Any violation of this article shall be deemed a misdemeanor punishable by a fine of two hundred dollars, six months' imprisonment or both.

SANITARY CODE

CHAPTER IV

Regulation 4. Distribution of living cultures of pathogenic bacteria. —No person having in his possession cultures of pathogenic microörganisms or viruses other than vaccine virus shall sell or convey such cultures to any other person or to any laboratory unless such sale or conveyance shall have been approved by the state commissioner of health.

APPENDIX C

OUTFITS FOR THE SUBMISSION OF SPECIMENS

POSTAL LAWS AND REGULATIONS

Specimens to be mailed must be enclosed in cases that meet the requirements of the Postal Laws and Regulations. Specifications of outfits that have been found satisfactory and that conform to these regulations are described.

POSTAL LAWS AND REGULATIONS GOVERNING THE SHIPMENT OF DISEASED TISSUES, BLOOD, SERUM, AND CULTURES OF PATHOGENIC MICROORGANISMS

Section 589. (**a**) Specimens of diseased tissues, blood, serum, and cultures of pathogenic microorganisms may be admitted to the mail for transmission to United States, State, municipal, or other laboratories in possession of permits referred to in paragraph 2 of this section only when enclosed in mailing cases constructed in accordance with this regulation, provided that bacteriologic or unfixed pathologic specimens of plague and cholera shall not be admitted to the mails except when prepared as hereinafter specifically provided.

(**b**) Pathologic specimens of plague and cholera which have been immersed for at least 72 hours in four times their volume of 4 per cent formaldehyde gas in water, or other fluid of equal or superior disinfecting power for a period sufficient to fix or harden the central portions of the specimen, may be admitted to the mails if packed in the same manner as herein prescribed for other unfixed pathologic tissues (par. 3, subpars. a, b, c).

(**c**) Cultures and infectious material of plague, cholera, anthrax, undulant fever, and tularaemia may be admitted to the mails if enclosed in stout glass tubes sealed by fusion of the glass and packed in a larger stout glass container with a layer of absorbent cotton soaked in 4 per cent formaldehyde surrounding the inner container. The outer glass container shall be closed with a rubber stopper or cork of good quality or by fusion of the glass. This double glass container shall then be packed in accordance with the provisions of paragraph 3, subparagraphs b and c.

2. No package containing diseased tissue, blood, serum, or cultures of pathogenic microorganisms shall be delivered to any representative of any of the said laboratories until a permit shall have first been issued by the Postmaster General, certifying that said institution has been found to be entitled, in accordance with the requirements of this regulation, to receive such specimens.

3. (a) Specimens of sputum, feces, pus, unfixed diseased tissue, or other infectious material fluid in nature or shipped with nondisinfecting fluid shall be placed in stout glass containers of suitable size (but not more than 3 inches in diameter) closed with a metal cover with a rubber, cork, or paraffined paper leak-proof washer or with a cork or rubber stopper of good quality or by fusing the glass, provided that large fixed specimens of diseased tissue may be prepared for shipment outside of mail bags when packed in accordance with the provisions of subparagraph b.

(b) The aforesaid glass container shall then be placed in a cylindrical sheet-metal box, with soldered joints, closed by a metal screw cover with a rubber or felt washer. The vial or test tube in this sheet-metal box shall be completely and evenly surrounded by absorbent cotton or other suitable absorbent in quantity sufficient to absorb the contents of the glass container should it be broken.

> Paragraph 3 (b) was amended for the duration of the war, to read as follows:
>
> (b) The aforesaid glass container shall then be placed in (1) a cylindrical sheet-metal box, with soldered joints, closed by a metal screw cover, or (2) a paraffin impregnated heavy cardboard container with ends made of metal, or a suitable substitute for metal and/or cardboard. A sleeve type of closure may be employed provided that the overlap is at least one-third the length of the cylinder and in any case at least two inches. The closure shall be sealed with tape. Or, (3) a one-piece bored wooden cylinder at least three-sixteenths of an inch thick in its thinnest part with a threaded screw top.
>
> The screw top covers shall be provided with rubber or felt washers and shall be threaded with sufficient screw threads to require at least one and one-half full turns before they will come off.
>
> The vial or test tube in the above containers shall be completely and evenly surrounded by absorbent cotton or other suitable absorbent in quantity sufficient to absorb the contents of the glass containers, should it be broken. (*Order No. 17516, April 21, 1942. Postal Bulletin No. 18430, April 29, 1942.*)

(c) The sheet-metal box with its contents shall then be enclosed in a closely fitting wooden or papier-mâché box or tube, at least three-sixteenths of an inch thick in its thinnest part or in a sheet-metal box or tube of sufficient strength to resist rough handling and support the weight of the mails piled in bags. This tube shall be tightly closed with a screw-top cover with sufficient screw threads to require at least one and one-half full turns before it will come off.

(d) Cultures in solid media, blood, serum, spinal fluid, fixed and completely disinfected diseased tissue and infectious materials on swabs shall be transmitted in a stout glass container of suitable size (but not more than 3 inches in diameter) closed with a metal cover with a rubber, cork, or paraffined paper washer or with a stopper of rubber, paraffined cork, or cotton, the last sealed with paraffin or covered with a tightly fitting rubber cap. The tube shall then be packed in a single wooden or papier-mâché cylindrical box or tube, at least three-sixteenths of an inch thick in its thinnest part or in a sheet-metal box or tube, of sufficient strength to resist rough handling and support the weight of the mails piled in bags. The glass container in this box or tube shall be completely and evenly surrounded by absorbent cotton or other suitable absorbent packing material. Cultures in media that are fluid at the ordinary temperature (below 45°C. or 113°F.) may be mailed if packed in stout glass vials closed by fusing the glass and enclosed as in subparagraphs (b) and (c).

(e) Specimens of blood dried on glass microscopic slides for the diagnosis of malaria or typhoid fever by the Widal test or of other conditions shall be sent in any strong mailing case which is not liable to breakage or loss of the specimen in transit.

(f) Large pathological specimens of fixed diseased tissue and shipments of large numbers of small specimens may be prepared for shipment outside of mail bags. Small specimens of sputum, blood, serum, spinal fluid, pus, feces, fixed or unfixed diseased tissue, or other material fluid in nature or shipped with fluid, forming part of such a shipment shall be placed in stout glass containers as in subparagraph (a) and individually evenly wrapped in absorbent cotton or other suitable absorbent material in sufficient quantity to absorb all the fluid in case of breakage. Large specimens of fixed diseased tissue shall be placed in securely sealed glass containers or in securely closed (hermetically sealed or screw-top or approved patent-top) metal containers with the necessary preservative fluid. The container shall be surrounded by sawdust or other suitable absorbent material to protect against breakage or leakage. Small and large specimens so prepared shall be shipped in a strong securely closed box marked "Fragile—Liquid. This Side Up"; or with similar inscription, and be transported outside of mail bags.

4. Upon the outside of every package of diseased tissue, blood, serum, or cultures

of pathogenic microorganisms admitted to the mails shall be written or printed the words "Specimen for bacteriological examination. This package shall be pouched with letter mail." Except that large specimens or shipments prepared under paragraph 3, subparagraph (f) shall be marked "Specimen for bacteriologic examination." (Effective October 5, 1935.)

SPECIMENS FOR DIAGNOSTIC EXAMINATION

Colors and letters have been used to aid physicians in selecting the appropriate outfit and to facilitate the handling of specimens upon arrival at the laboratory. For example, the label on the container for the submission of specimens from diphtheria is blue and is marked "D." The history form enclosed in the outfit is also blue. In some instances where colored paper is not available, the label is white with printing the color of the history form. The address label bears the note:

Postmaster **This Package to be Pouched with LETTER MAIL**
Specimens for Bacteriologic Examination
(P. L. & R. §589)
THIRD CLASS RATE: 1 TO 8 OZ., $1\frac{1}{2}$ C. EA. 2 OZ; OVER 8 OZ., FOURTH CLASS RATE

Diphtheria Culture Outfit

Purpose.—Submission of cultures to be examined for diphtheria bacilli—label, blue, lettered "D."

Mailing case, double.—*Outer container.* Fiber with threaded metal rim, $5\frac{3}{4}$ by $1\frac{3}{8}$ in. inside dimensions; bottom metal; indented paper pad for lining metal screw cover. *Inner container.* Tin, $5\frac{1}{2}$ by $1\frac{1}{4}$ in. outside dimensions; corrugated paper pad lining, corrugated paper pad for lining bottom, and felt pad for metal screw cover.

Test tube.—125 by 12.5–14 mm., containing coagulated-serum medium (*F15*), plugged with nonabsorbent cotton and stoppered with No. $2\frac{1}{2}$ paraffined cork, sealed with paraffin. White printed label for identification of patient:

Patient

Do not use if seal is broken

FIG. 77

Swab.—Absorbent cotton on 16-gauge wire $5\frac{1}{8}$ in. long; enclosed in test tube, 100 by 7–8 mm., plugged with nonabsorbent cotton.

Forms.—History form, blue, 5 by 8 in., and directions for collecting specimen folded and wrapped around the tubes. See figures 78a, 78b, 79 and 80.

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NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY 1

EDWARD S. GODFREY, JR., M.D., *Commissioner*

DIVISION OF LABORATORIES AND RESEARCH

GILBERT DALLDORF, M.D.
Director
MARY B. KIRKBRIDE, SC.D.
Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

DIPHTHERIA

EXAMINATION OF CULTURE

(Directions for taking cultures are given on accompanying sheet)

Number of culture, 1, 2, 3, etc.................... Date taken....................

Results of previous examinations....................

Purpose for which specimen was taken:

Diagnosis.................... Release.................... To determine carrier....................

Culture from throat....................or nose....................

Patient.................... Age....................

Address....................
(City, town or village) (County)

Occupation.................... Sex.................... Color....................

Clinical diagnosis....................

Condition of throat....................and nose....................

Condition of tonsils....................

Location of membrane....................

Duration of disease.................... Date of onset....................

Has antitoxin been administered.................... Date....................

Has the patient been in contact with a known case of diphtheria or with a convalescent carrier of diphtheria bacilli....................

Have any carriers been traced to this patient....................

Other information concerning specimen....................

....

....

Name and address of physician Name and address of health officer

....

....

....

Laboratory number.................... Examined....................

Received.................... Examiner....................

Result of examination....................

(OVER)

FIG. 78a. (Blue) (Front)

RECORD OF VIRULENCE TEST

Type of microorganism in original film ....

....

....

Plated	Fished	Plated	Fished

Sugar Reaction				Intracutaneous Test				
Date	Dextrose	Saccharose	Dextrin	Date	Pig No.	24 hrs.	48 hrs.	72 hrs.

Laboratory number M.................................... Exam. completed....................

Examiner..

Result of examination...

....

....

....

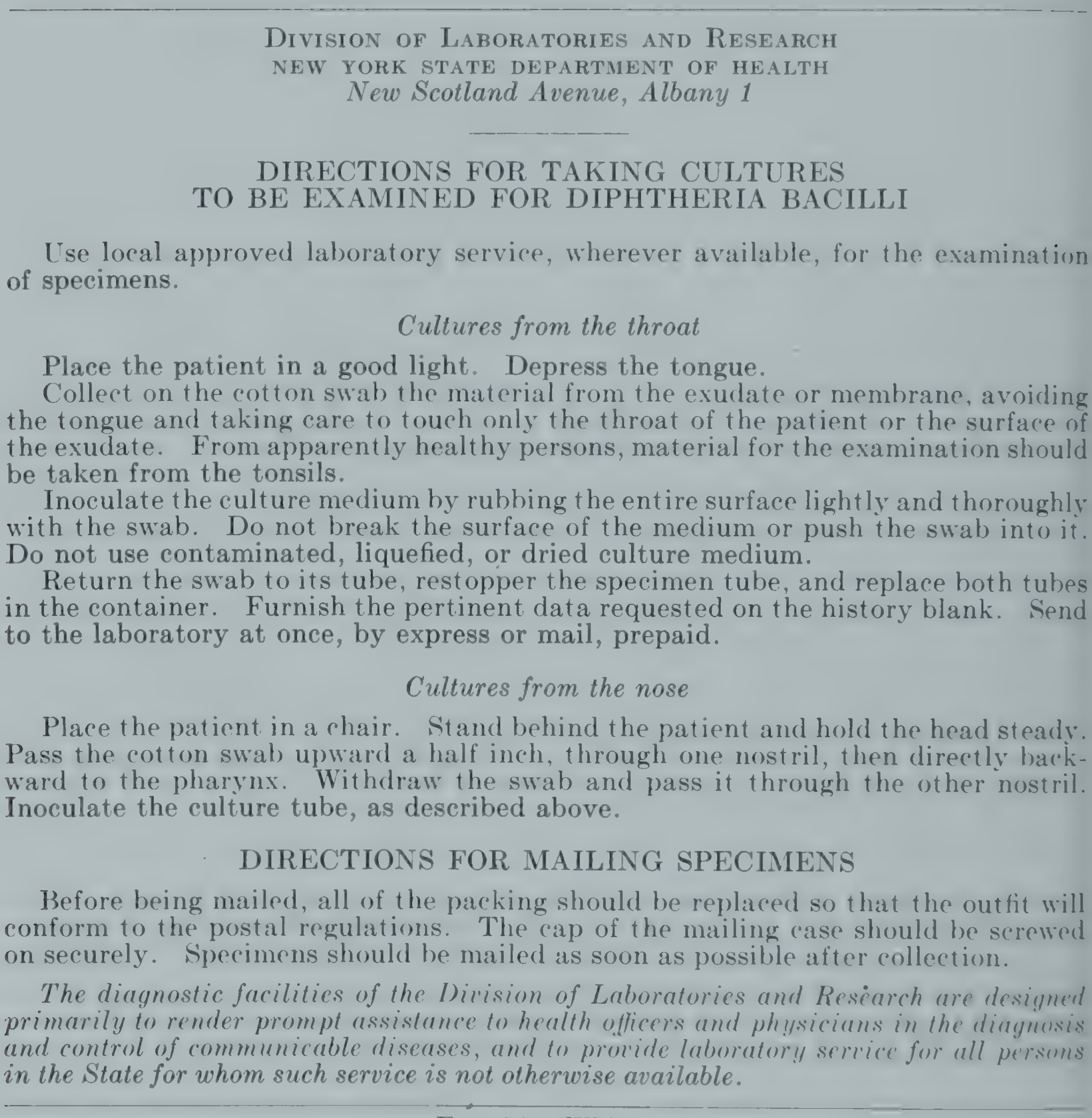
....

Specimen opened by.................... Checked for virulence test by............................

Original film made by..

Typed report sent on..

FIG. 78b. (Blue) (Back)



Division of Laboratories and Research
NEW YORK STATE DEPARTMENT OF HEALTH
New Scotland Avenue, Albany 1

DIRECTIONS FOR TAKING CULTURES TO BE EXAMINED FOR DIPHTHERIA BACILLI

Use local approved laboratory service, wherever available, for the examination of specimens.

Cultures from the throat

Place the patient in a good light. Depress the tongue.

Collect on the cotton swab the material from the exudate or membrane, avoiding the tongue and taking care to touch only the throat of the patient or the surface of the exudate. From apparently healthy persons, material for the examination should be taken from the tonsils.

Inoculate the culture medium by rubbing the entire surface lightly and thoroughly with the swab. Do not break the surface of the medium or push the swab into it. Do not use contaminated, liquefied, or dried culture medium.

Return the swab to its tube, restopper the specimen tube, and replace both tubes in the container. Furnish the pertinent data requested on the history blank. Send to the laboratory at once, by express or mail, prepaid.

Cultures from the nose

Place the patient in a chair. Stand behind the patient and hold the head steady. Pass the cotton swab upward a half inch, through one nostril, then directly backward to the pharynx. Withdraw the swab and pass it through the other nostril. Inoculate the culture tube, as described above.

DIRECTIONS FOR MAILING SPECIMENS

Before being mailed, all of the packing should be replaced so that the outfit will conform to the postal regulations. The cap of the mailing case should be screwed on securely. Specimens should be mailed as soon as possible after collection.

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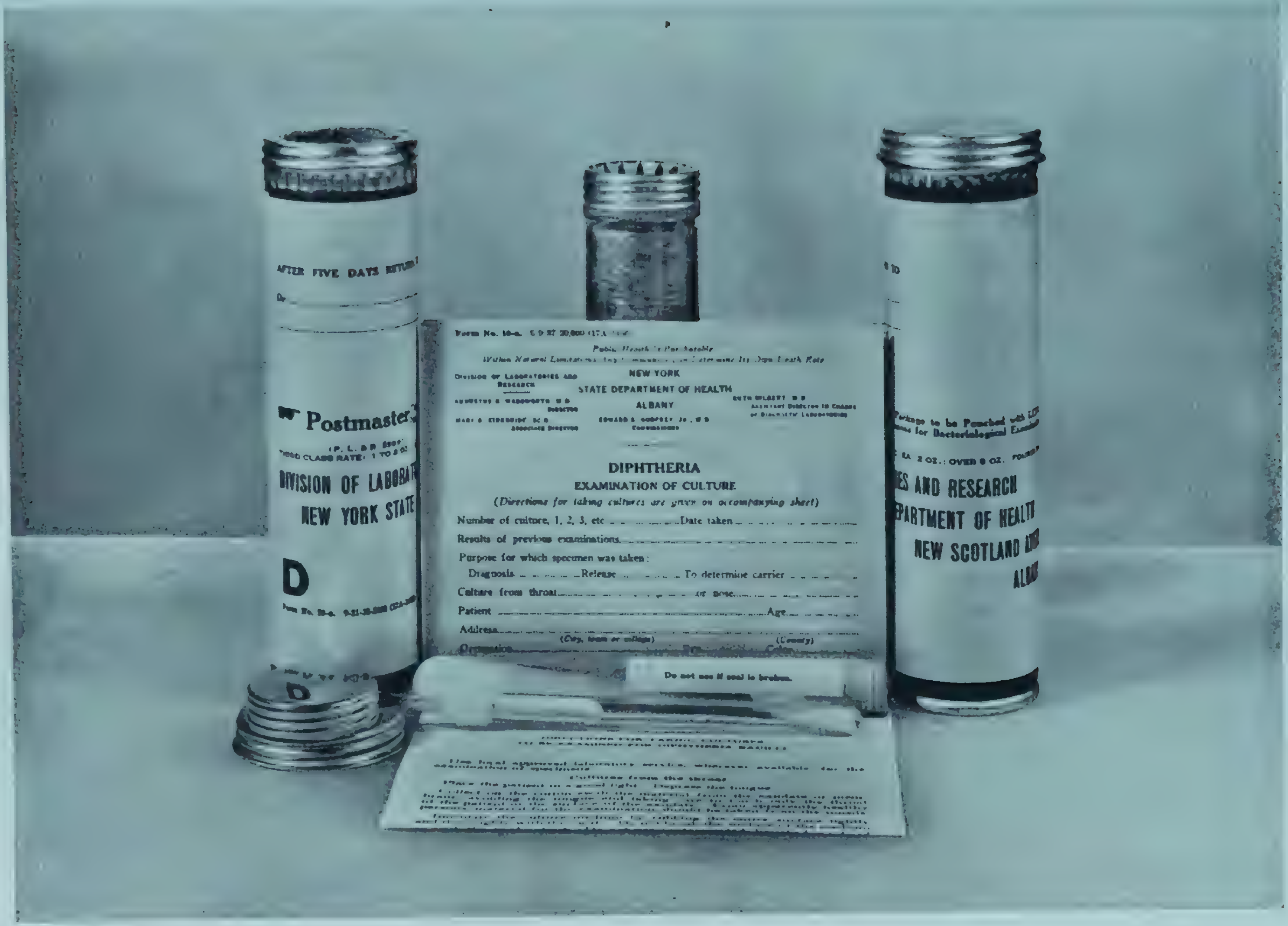
Fig. 79. (White)


AFTER FIVE DAYS RETURN
Postmaster
THIRD CLASS RATE
DIVISION OF LABORAT
NEW YORK STATE
D
D
Package to be Punched with
for Bacteriological Exam
ES AND RESEARCH
PARTMENT OF HEALT
NEW SCOTLAND
ALBA
Do not use if seal is broken.

NEW YORK
STATE DEPARTMENT OF HEALTH
ALBANY

DIPHTHERIA
EXAMINATION OF CULTURE
(*Directions for taking cultures are given on accompanying sheet*)

Number of culture, 1, 2, 3, etc ....Date taken ....
Results of previous examinations............
Purpose for which specimen was taken:
Diagnosis ....Release ....To determine carrier ....
Culture from throat............or nose............
Patient ....Age............
Address............
(City, town or village) (County)

FIG. 80. DIPHTHERIA CULTURE OUTFIT

Gonorrhea Culture Outfit

Purpose.—Submission of specimens for cultural and microscopic examination for gonococci—label, buff, lettered "V."

Mailing case.—Fiber with threaded metal rim, 6 by $3\frac{1}{8}$ in. inside dimensions; corrugated paper lining and pad for lining metal bottom; felt pad for lining metal screw cover. Whipcord, 8 ply, around each tube and cellucotton for packing at top, bottom, and sides of tubes and glass slides.

Test tubes.—Two, 133 by 15.5–16.5 mm., stoppered with No. $4\frac{1}{2}$ or No. 5 paraffined corks, containing coagulated-blood gelatin-agar medium (*F55.5B*). White printed label for identification of patient affixed so that one end extends onto the cork.

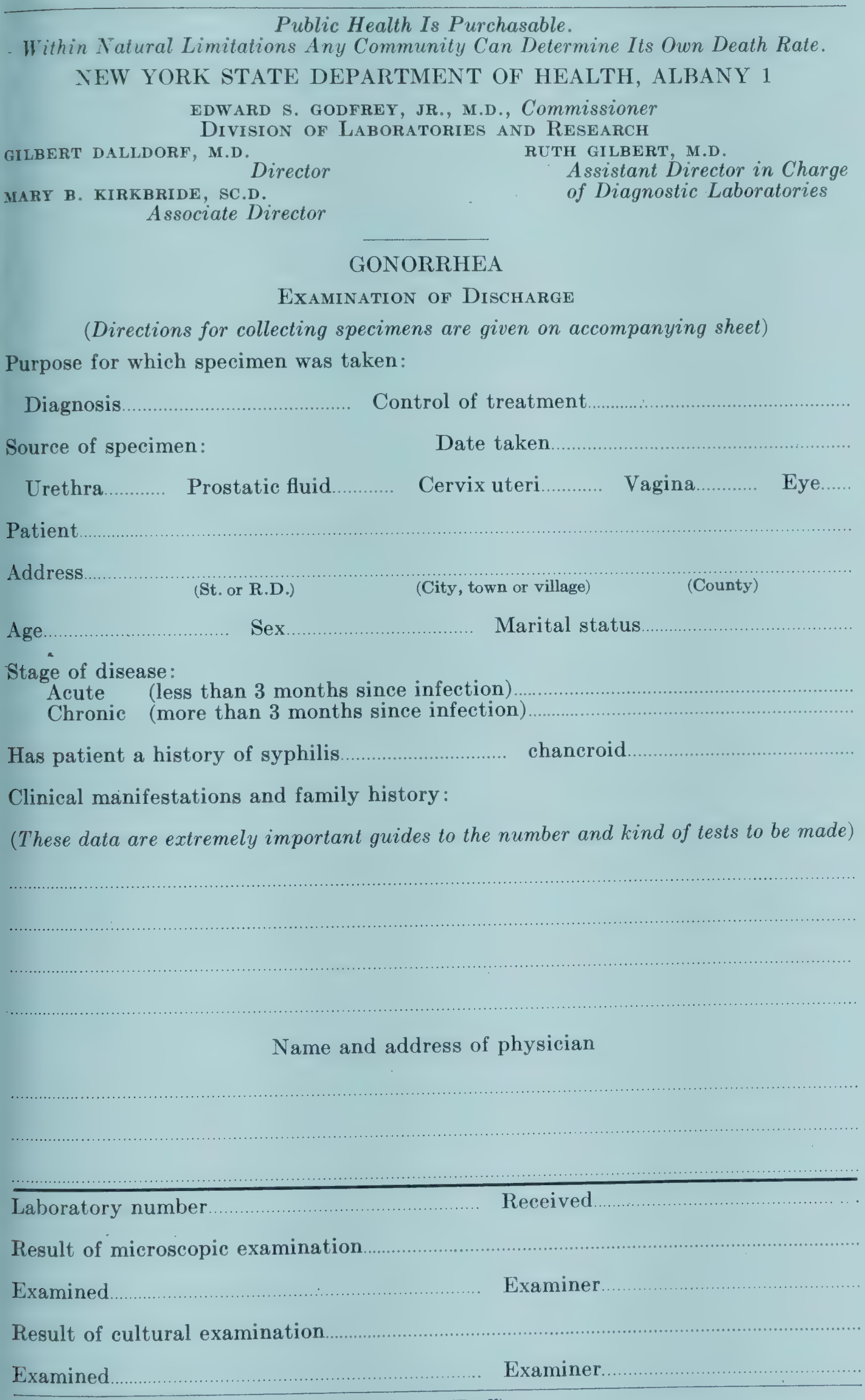
Swabs.—4, absorbent cotton, on 16-gauge wires $5\frac{1}{8}$ in. long, inserted into No. $4\frac{1}{2}$ or No. 5 paraffined corks and stoppered in tubes, 133 by 15.5–16.5 mm. White printed label for identification of patient affixed so that one end extends onto the cork.

Slides.—2, glass, 3 by 1 in., etched $\frac{1}{2}$ in. on one end with Etch-O-Lite; enclosed in a wooden box $3\frac{1}{8}$ by $1\frac{5}{16}$ by $\frac{9}{16}$ in.; printed directions secured to the box with cord:

To avoid breakage and loss secure
slides firmly in box before returning.

Fig. 81

Forms.—History form and direction sheet, 5 by 8 in., folded in quarters and placed close to the inside wall of the mailing case. See figures 82, 83a, 83b and 84.



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Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

GONORRHEA

EXAMINATION OF DISCHARGE

(Directions for collecting specimens are given on accompanying sheet)

Purpose for which specimen was taken:

Diagnosis.................... Control of treatment....................

Source of specimen: Date taken....................

Urethra.......... Prostatic fluid.......... Cervix uteri.......... Vagina.......... Eye......

Patient....................

Address....................
(St. or R.D.) (City, town or village) (County)

Age.................... Sex.................... Marital status....................

Stage of disease:
Acute (less than 3 months since infection)....................
Chronic (more than 3 months since infection)....................

Has patient a history of syphilis.................... chancroid....................

Clinical manifestations and family history:

(These data are extremely important guides to the number and kind of tests to be made)

....
....
....
....

Name and address of physician

....
....
....

Laboratory number.................... Received....................

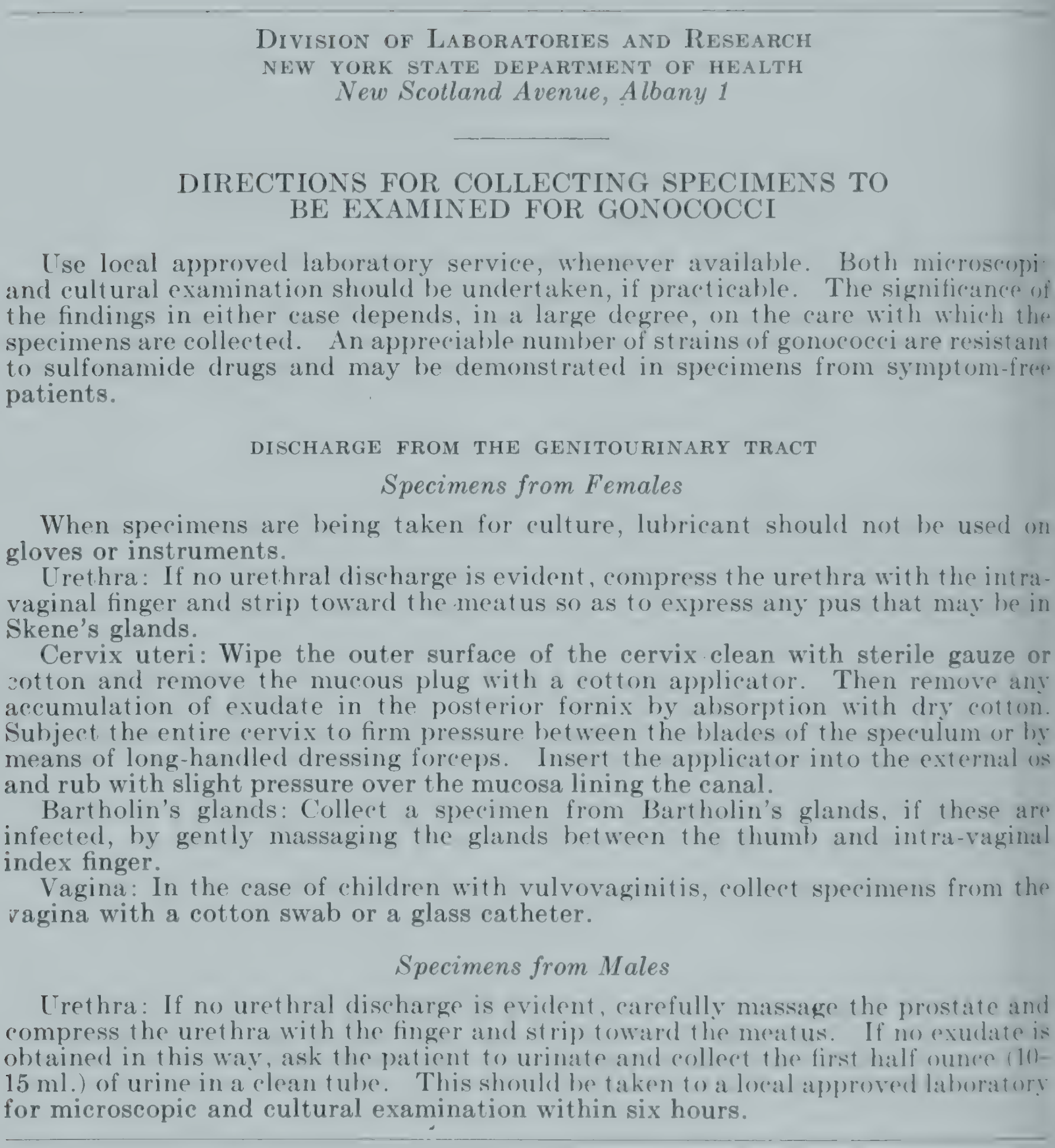
Result of microscopic examination....................

Examined.................... Examiner....................

Result of cultural examination....................

Examined.................... Examiner....................

FIG. 82. (Buff)



Division of Laboratories and Research
NEW YORK STATE DEPARTMENT OF HEALTH
New Scotland Avenue, Albany 1

DIRECTIONS FOR COLLECTING SPECIMENS TO BE EXAMINED FOR GONOCOCCI

Use local approved laboratory service, whenever available. Both microscopi and cultural examination should be undertaken, if practicable. The significance of the findings in either case depends, in a large degree, on the care with which the specimens are collected. An appreciable number of strains of gonococci are resistant to sulfonamide drugs and may be demonstrated in specimens from symptom-free patients.

DISCHARGE FROM THE GENITOURINARY TRACT

Specimens from Females

When specimens are being taken for culture, lubricant should not be used on gloves or instruments.

Urethra: If no urethral discharge is evident, compress the urethra with the intravaginal finger and strip toward the meatus so as to express any pus that may be in Skene's glands.

Cervix uteri: Wipe the outer surface of the cervix clean with sterile gauze or cotton and remove the mucous plug with a cotton applicator. Then remove any accumulation of exudate in the posterior fornix by absorption with dry cotton. Subject the entire cervix to firm pressure between the blades of the speculum or by means of long-handled dressing forceps. Insert the applicator into the external os and rub with slight pressure over the mucosa lining the canal.

Bartholin's glands: Collect a specimen from Bartholin's glands, if these are infected, by gently massaging the glands between the thumb and intra-vaginal index finger.

Vagina: In the case of children with vulvovaginitis, collect specimens from the vagina with a cotton swab or a glass catheter.

Specimens from Males

Urethra: If no urethral discharge is evident, carefully massage the prostate and compress the urethra with the finger and strip toward the meatus. If no exudate is obtained in this way, ask the patient to urinate and collect the first half ounce (10–15 ml.) of urine in a clean tube. This should be taken to a local approved laboratory for microscopic and cultural examination within six hours.

Fig. 83a. (White) (Front)

MISCELLANEOUS SPECIMENS

Gonococci may be demonstrated in discharge from the conjunctiva, the Fallopian tubes, pelvic lesions, rectal discharge, and occasionally in blood and joint and cerebrospinal fluids.

SUBMISSION OF SPECIMENS TO THE LABORATORY

For microscopic examination: Prepare thin films on glass slides by rolling swabs on which specimens have been collected over the surfaces of the slides or by using the edge of another slide as a spreader. The films should be thin and evenly spread. Allow films to dry thoroughly in the air without heating before replacing the slides in the box, specimen sides together.

For cultural examination: Entirely reliable procedures have not yet been developed for transporting by mail specimens to be cultured for gonococci. These examinations should, if possible, be made in a near-by approved laboratory promptly after collection. If this cannot be done, secure from the director of such a laboratory or his representative suitable medium and directions for its use.

When arrangements cannot be made to have the cultural examination undertaken in a near-by approved laboratory, swabs with discharge may be sent to the central laboratory in Albany in medium supplied in a special outfit for this purpose. Separate sterile swabs are provided for making slide preparations and for cultures. After preparing a film on a slide, replace the swab in the tube in which it was received. Collect discharge for cultural examination on another swab and thrust this into the medium and leave it there. Do not use medium that is dried, liquefied, or contaminated.

Fill out the information blank in detail.

The diagnostic facilities of the Division of Laboratories and Research are designed primarily to render prompt assistance to health officers and physicians in the diagnosis and control of communicable diseases, and to provide laboratory service for all persons in the state for whom such service is not otherwise available.

FIG. 83b. (White) (Back)

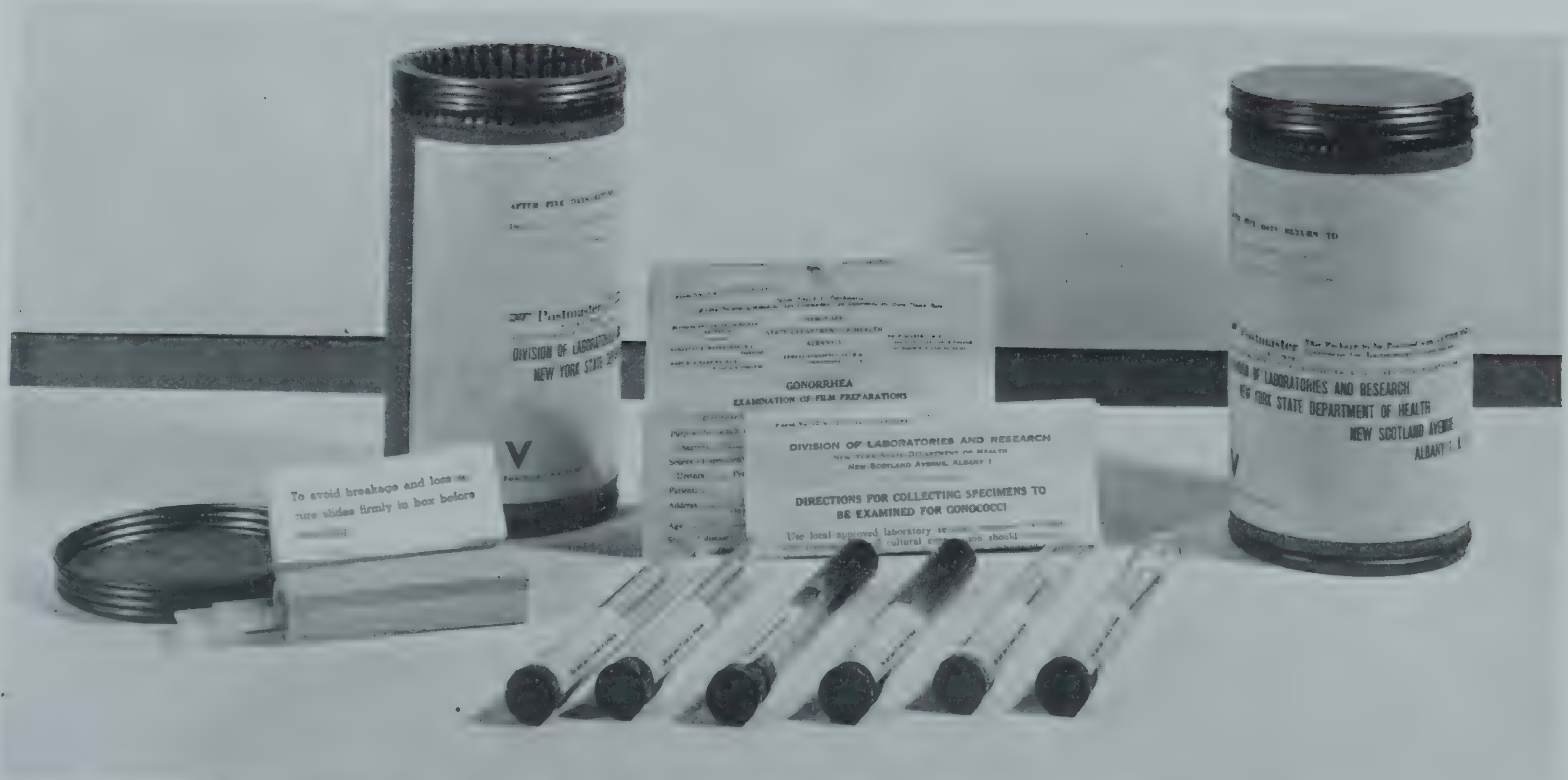

Postmaster
DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
NEW SCOTLAND AVENUE
ALBANY 1
GONORRHEA
EXAMINATION OF FILM PREPARATIONS
DIVISION OF LABORATORIES AND RESEARCH
NEW SCOTLAND AVENUE, ALBANY 1
DIRECTIONS FOR COLLECTING SPECIMENS TO BE EXAMINED FOR GONOCOCCI
To avoid breakage and loss secure slides firmly in box before

FIG. 84. GONORRHEA CULTURE OUTFIT

Jar Outfit (1 oz.)

Purpose.—Submission of: (1) Feces or urine to be examined for bacillary incitants of enteric disease—label, green, marked "T"; (2) Specimens for miscellaneous examinations and tissue for microscopic examination—label, pink, lettered "M."

Mailing case, double.—*Outer container.* Fiber with threaded metal rim, $3\frac{1}{2}$ by 2 in. inside dimensions; corrugated paper pad for lining metal bottom and indented paper pad for metal screw cover. *Inside container.* Tin, $3\frac{1}{4}$ by $1\frac{3}{4}$ in. outside dimensions; corrugated paper pad lining and corrugated paper pad for lining bottom and felt pad for metal screw cover. Cellucotton for packing at top and bottom of jar inserted in inside container.

Jar.—Glass, 1 oz., round, wide-mouthed with metal screw cap. White printed label for identification of patient affixed:

> Patient
>
> Screw cover on tightly
> to prevent leakage

FIG. 85

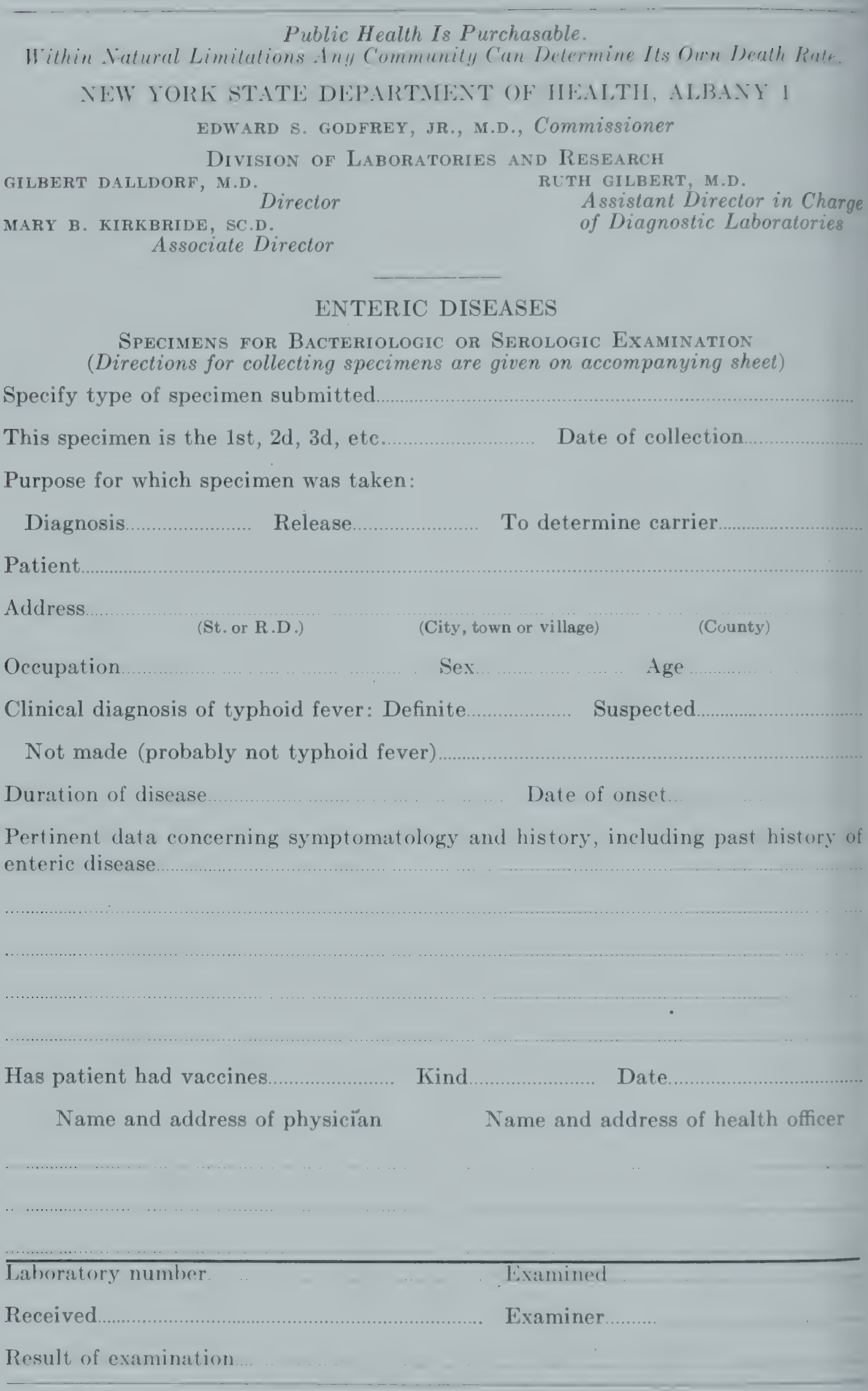
For collection of feces and urine, purpose (1), jar containing approximately 10-ml buffered 30-per-cent glycerol solution (*F70*).

Swab.—Absorbent cotton on wooden stick $2\frac{3}{4}$ in. long, wrapped in paraffin paper and inserted between jar and inside container for feces.

Forms.—History form, 5 by 8 in., and directions for the collection of specimen folded and wrapped around inside containers. See figures 86a, 86b, 87a, 87b, 88, 89, 90a, 90b, 90c, 90d and 91. Also enclosed, the following printed directions:

> Protect the laboratory worker.
>
> Place the history slip around the tin container. If it is placed around the tube or jar, it may become contaminated.

FIG. 92



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Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

ENTERIC DISEASES

SPECIMENS FOR BACTERIOLOGIC OR SEROLOGIC EXAMINATION
(*Directions for collecting specimens are given on accompanying sheet*)

Specify type of specimen submitted..

This specimen is the 1st, 2d, 3d, etc.................... Date of collection....................

Purpose for which specimen was taken:

Diagnosis.................... Release.................... To determine carrier....................

Patent..

Address..
(St. or R.D.) (City, town or village) (County)

Occupation.................... Sex.................... Age....................

Clinical diagnosis of typhoid fever: Definite.................... Suspected....................

Not made (probably not typhoid fever)..

Duration of disease.................... Date of onset....................

Pertinent data concerning symptomatology and history, including past history of enteric disease..

....

....

....

....

Has patient had vaccines.................... Kind.................... Date....................

Name and address of physician Name and address of health officer

....

....

....

Laboratory number.................... Examined....................

Received.................... Examiner....................

Result of examination..

FIG. 86a. (Green) (Front)

Laboratory number .... Opened by ....

Patient .... Plated by ....

Material .... Fished by ....

Date fished	Sugar reactions of fishings from			
	Endo	Bismuth sulfite		D-citrate

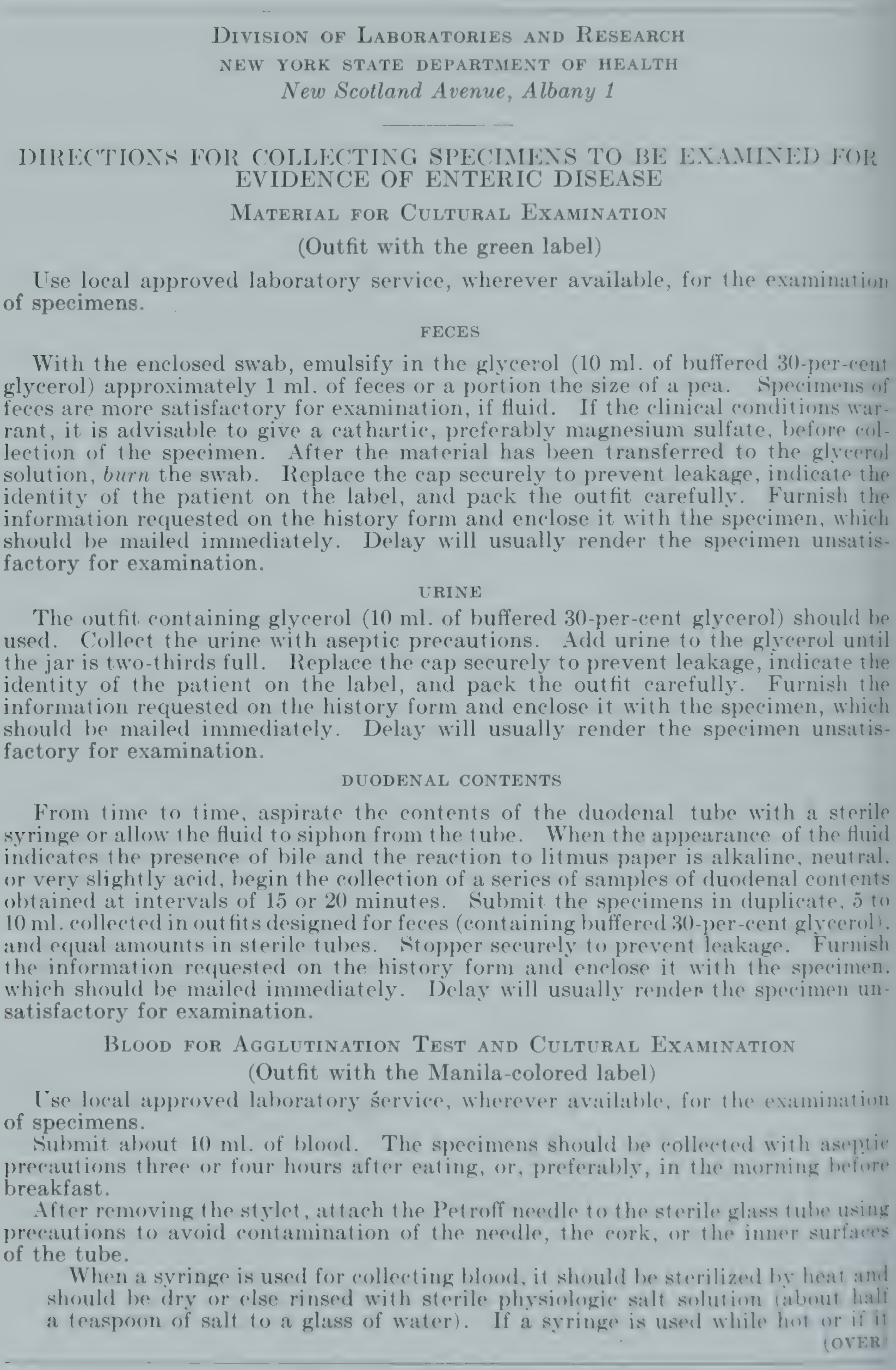
Fishing	Macroscopic slide agglutination test			Morphology	
	Normal serum	Typhoid serum	Multivalent dysentery serum	Gram stain	Motility

Macroscopic tube agglutination test

Serum	Suspension	Date	Dilutions							Negative control	Positive control

Typed report sent on ....

FIG. 86b. (Green) (Back)



Division of Laboratories and Research
NEW YORK STATE DEPARTMENT OF HEALTH
New Scotland Avenue, Albany 1

DIRECTIONS FOR COLLECTING SPECIMENS TO BE EXAMINED FOR EVIDENCE OF ENTERIC DISEASE

Material for Cultural Examination

(Outfit with the green label)

Use local approved laboratory service, wherever available, for the examination of specimens.

FECES

With the enclosed swab, emulsify in the glycerol (10 ml. of buffered 30-per-cent glycerol) approximately 1 ml. of feces or a portion the size of a pea. Specimens of feces are more satisfactory for examination, if fluid. If the clinical conditions warrant, it is advisable to give a cathartic, preferably magnesium sulfate, before collection of the specimen. After the material has been transferred to the glycerol solution, *burn* the swab. Replace the cap securely to prevent leakage, indicate the identity of the patient on the label, and pack the outfit carefully. Furnish the information requested on the history form and enclose it with the specimen, which should be mailed immediately. Delay will usually render the specimen unsatisfactory for examination.

URINE

The outfit containing glycerol (10 ml. of buffered 30-per-cent glycerol) should be used. Collect the urine with aseptic precautions. Add urine to the glycerol until the jar is two-thirds full. Replace the cap securely to prevent leakage, indicate the identity of the patient on the label, and pack the outfit carefully. Furnish the information requested on the history form and enclose it with the specimen, which should be mailed immediately. Delay will usually render the specimen unsatisfactory for examination.

DUODENAL CONTENTS

From time to time, aspirate the contents of the duodenal tube with a sterile syringe or allow the fluid to siphon from the tube. When the appearance of the fluid indicates the presence of bile and the reaction to litmus paper is alkaline, neutral, or very slightly acid, begin the collection of a series of samples of duodenal contents obtained at intervals of 15 or 20 minutes. Submit the specimens in duplicate, 5 to 10 ml. collected in outfits designed for feces (containing buffered 30-per-cent glycerol), and equal amounts in sterile tubes. Stopper securely to prevent leakage. Furnish the information requested on the history form and enclose it with the specimen, which should be mailed immediately. Delay will usually render the specimen unsatisfactory for examination.

Blood for Agglutination Test and Cultural Examination

(Outfit with the Manila-colored label)

Use local approved laboratory service, wherever available, for the examination of specimens.

Submit about 10 ml. of blood. The specimens should be collected with aseptic precautions three or four hours after eating, or, preferably, in the morning before breakfast.

After removing the stylet, attach the Petroff needle to the sterile glass tube using precautions to avoid contamination of the needle, the cork, or the inner surfaces of the tube.

When a syringe is used for collecting blood, it should be sterilized by heat and should be dry or else rinsed with sterile physiologic salt solution (about half a teaspoon of salt to a glass of water). If a syringe is used while hot or if it

(OVER

Fig. 87a. (White) (Front)

contains an antiseptic, water, or ether, the blood may become hemolyzed. The blood should be transferred to the sterile test tube immediately.

Allow the tube to remain in a slanting position for one-half hour at room temperature. If the specimen cannot be sent to the laboratory immediately, keep it in a cool place but at a temperature above freezing, since freezing will cause hemolysis of the blood.

Furnish the information requested on the history form and mail it with the specimen.

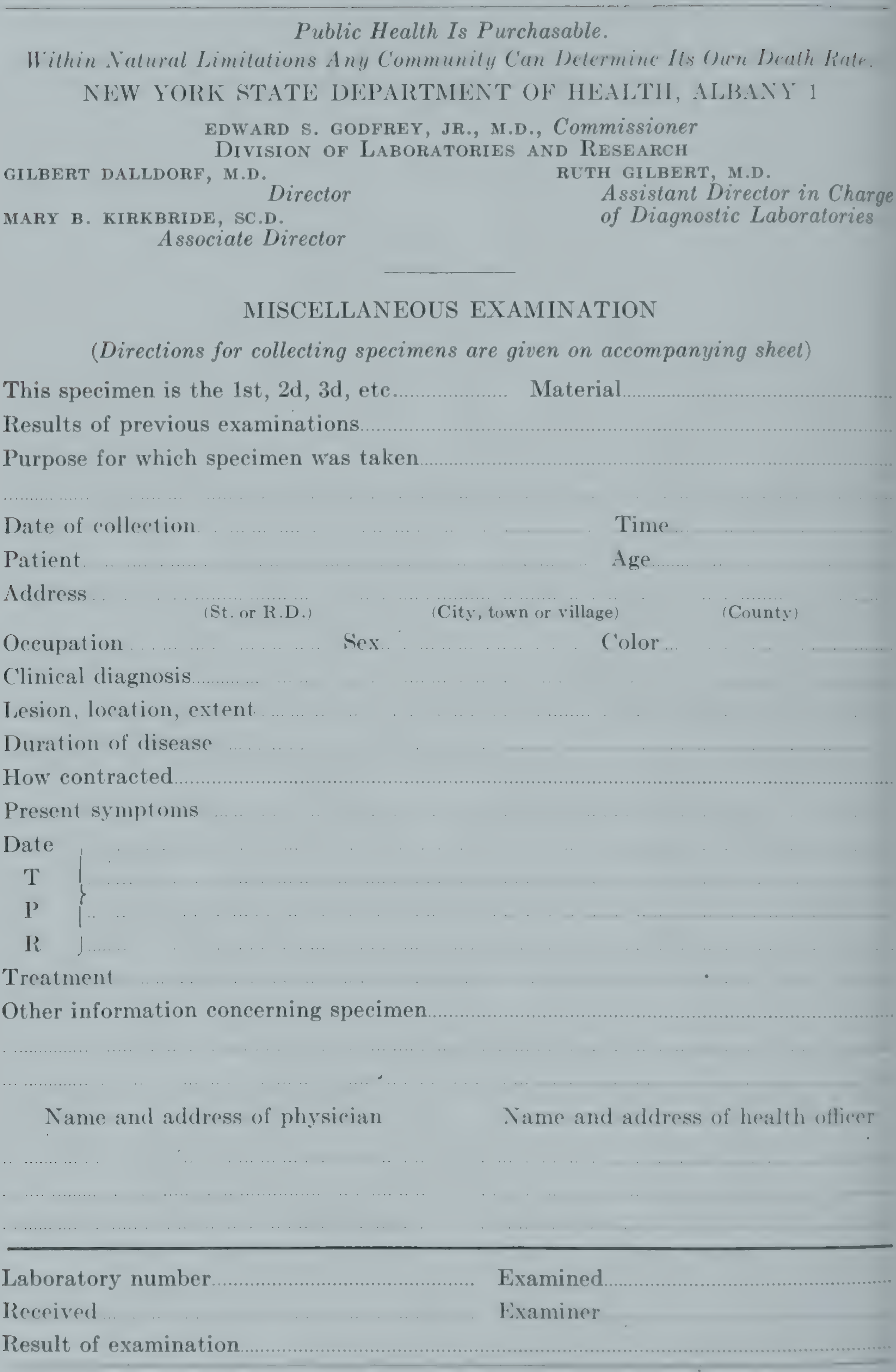
Enclose the used needle in the envelope provided and return with the specimen

DIRECTIONS FOR MAILING SPECIMENS

Before being mailed, all of the packing should be replaced so that the outfit will conform to the postal regulations. The cap of the mailing case should be screwed on securely. Specimens should be mailed as soon as possible after collection.

The diagnostic facilities of the Division of Laboratories and Research are designed primarily to render prompt assistance to health officers and physicians in the diagnosis and control of communicable diseases, and to provide laboratory service for all persons in the State for whom such service is not otherwise available.

Fig. 87b. (White) (Back)



Public Health Is Purchasable.

Within Natural Limitations Any Community Can Determine Its Own Death Rate.

NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY 1

EDWARD S. GODFREY, JR., M.D., *Commissioner*

DIVISION OF LABORATORIES AND RESEARCH

GILBERT DALLDORF, M.D.
Director

MARY B. KIRKBRIDE, SC.D.
Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

MISCELLANEOUS EXAMINATION

(*Directions for collecting specimens are given on accompanying sheet*)

This specimen is the 1st, 2d, 3d, etc.................... Material..

Results of previous examinations..

Purpose for which specimen was taken..

....

Date of collection.. Time..........................

Patient.. Age...........................

Address..
(St. or R.D.) (City, town or village) (County)

Occupation.............................. Sex.............................. Color..............................

Clinical diagnosis..

Lesion, location, extent..

Duration of disease..

How contracted..

Present symptoms..

Date..

T ....

P ....

R ....

Treatment..

Other information concerning specimen..

....

....

Name and address of physician Name and address of health officer

....

....

....

Laboratory number.. Examined..

Received.. Examiner...

Result of examination..

FIG. 88. (Pink)

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NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY 1

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MARY B. KIRKBRIDE, SC.D.
Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

EXAMINATION OF TISSUE

Date of collection

Patient

Address
(St. or R.D.) (City, town or village) (County)

Age Sex Marital status

Nature of specimen and exact anatomic location

Pertinent clinical data, including clinical diagnosis and information concerning X-ray and other laboratory findings. (*Please do not omit. This information may be essential to correct interpretation.*)

Name and address of physician

Laboratory number Examined

Received Examiner

Result of examination

FIG. 89. (White)

DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
New Scotland Avenue, Albany 1

DIRECTIONS FOR THE COLLECTION AND PREPARATION OF MISCELLANEOUS SPECIMENS

SPUTUM FOR PNEUMOCOCCUS TYPE DIFFERENTIATION

Use local approved laboratory service, wherever available, for the examination of specimens. If local laboratory facilities are not available, a specimen of sputum taken according to the directions specified, can be sent to the Division of Laboratories and Research. In pneumonia, prompt diagnosis and early institution of therapeutic measures are essential. If serum therapy is to be used, this fact should be indicated on the history form and the specimen should either be delivered by messenger or be sent by special delivery. Secure sputum coughed up from the deeper air passages. Specimens consisting chiefly of saliva or nasopharyngeal secretions are not satisfactory. When sputum cannot be obtained in the usual manner, as so frequently happens with children, induce the patient to cough, and as the secretion is forced forward, attempt to collect some of it from the throat on a sterile swab. Stomach contents of young children may also be found to contain sputum that has been swallowed.

In addition to the study of sputum, cultural tests of blood should be undertaken. The initial sputum specimen and blood culture should be obtained before serum therapy or chemotherapy is begun; otherwise, it is often difficult if not impossible to determine the incitant. The blood can be left at the laboratory by the same messenger who delivers the specimen of sputum. Collect the blood in a tube containing a suitable sterile solution of an anticoagulant, such as sodium citrate, that will not inhibit bacterial growth; or introduce it directly into fluid medium in an outfit for the purpose.

Furnish the information requested on the history form and send it with the specimen. Note especially the time and character of onset; that is, whether or not the patient had an initial chill, pain in the chest, and coughing. Make a record also of the morning and evening temperatures and pulse rate.

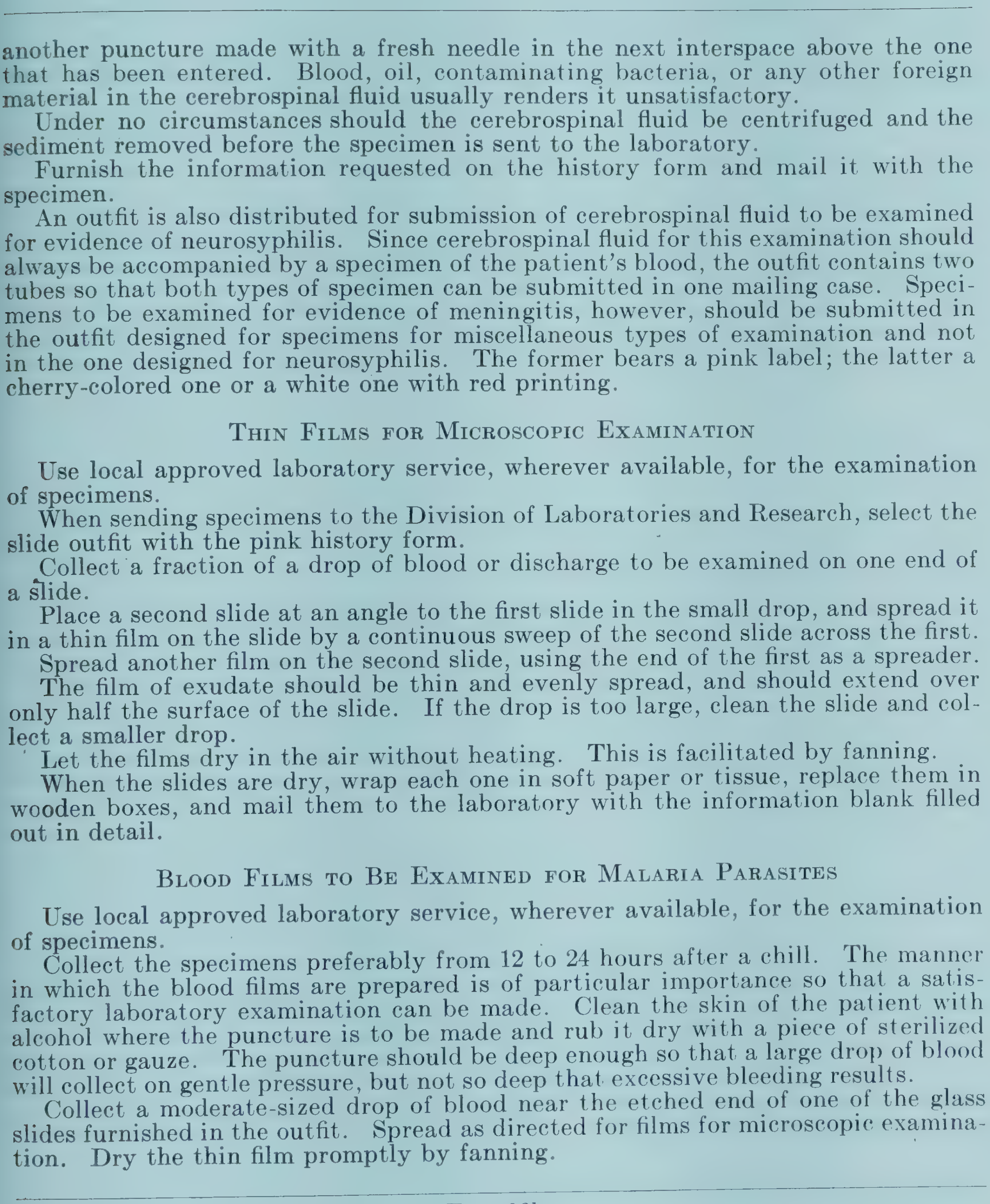
CEREBROSPINAL FLUID FOR CULTURAL EXAMINATION

Use local approved laboratory service, wherever available, for the examination of specimens.

If local laboratory facilities are not available, specimens of cerebrospinal fluid (at least 5 ml.) to be examined for evidence of meningitis should be sent without delay to the Division of Laboratories and Research in the tube outfit with the pink label. Remove the swab from the cork before restoppering the tube.

Proper collection of the specimen is of particular importance. When a lumbar puncture is made, two needles, thoroughly cleaned and sterilized in dry heat, should be available. They should have been carefully sharpened, since the use of a dull needle is usually responsible for admixture of blood in specimens of cerebrospinal fluid. If there is evidence of blood in the fluid, the tap should be discontinued and

FIG. 90a. (White) (Front)
(Continued in Figs. 90b–d)



another puncture made with a fresh needle in the next interspace above the one that has been entered. Blood, oil, contaminating bacteria, or any other foreign material in the cerebrospinal fluid usually renders it unsatisfactory.

Under no circumstances should the cerebrospinal fluid be centrifuged and the sediment removed before the specimen is sent to the laboratory.

Furnish the information requested on the history form and mail it with the specimen.

An outfit is also distributed for submission of cerebrospinal fluid to be examined for evidence of neurosyphilis. Since cerebrospinal fluid for this examination should always be accompanied by a specimen of the patient's blood, the outfit contains two tubes so that both types of specimen can be submitted in one mailing case. Specimens to be examined for evidence of meningitis, however, should be submitted in the outfit designed for specimens for miscellaneous types of examination and not in the one designed for neurosyphilis. The former bears a pink label; the latter a cherry-colored one or a white one with red printing.

Thin Films for Microscopic Examination

Use local approved laboratory service, wherever available, for the examination of specimens.

When sending specimens to the Division of Laboratories and Research, select the slide outfit with the pink history form.

Collect a fraction of a drop of blood or discharge to be examined on one end of a slide.

Place a second slide at an angle to the first slide in the small drop, and spread it in a thin film on the slide by a continuous sweep of the second slide across the first.

Spread another film on the second slide, using the end of the first as a spreader.

The film of exudate should be thin and evenly spread, and should extend over only half the surface of the slide. If the drop is too large, clean the slide and collect a smaller drop.

Let the films dry in the air without heating. This is facilitated by fanning.

When the slides are dry, wrap each one in soft paper or tissue, replace them in wooden boxes, and mail them to the laboratory with the information blank filled out in detail.

Blood Films to Be Examined for Malaria Parasites

Use local approved laboratory service, wherever available, for the examination of specimens.

Collect the specimens preferably from 12 to 24 hours after a chill. The manner in which the blood films are prepared is of particular importance so that a satisfactory laboratory examination can be made. Clean the skin of the patient with alcohol where the puncture is to be made and rub it dry with a piece of sterilized cotton or gauze. The puncture should be deep enough so that a large drop of blood will collect on gentle pressure, but not so deep that excessive bleeding results.

Collect a moderate-sized drop of blood near the etched end of one of the glass slides furnished in the outfit. Spread as directed for films for microscopic examination. Dry the thin film promptly by fanning.

FIG. 90b.

Prepare a thick film on the other slide. The margin of this film should be about one-fourth of an inch from the nonetched end of the slide. A thick film should consist of from 3 to 5 average-sized drops of blood. These may be deposited on an area about three-quarters of an inch in diameter and promptly combined with a needle or the corner of another slide; or the surface of the slide may be touched to a large drop of blood and the slide moved in narrow circles until a preparation of the size mentioned is obtained. The film must be fairly thick in order to have sufficient blood for adequate examination and must not be so thick that it will peel from the slide when it is being stained. When the thick blood film is still wet, ordinary printing can just be read through it. In order to protect the specimen from dust during drying, the slide should be supported with the film side down. A wooden slide container may be used for the purpose. It is important to have the slide level, since otherwise the blood might collect at one side of the film and not be evenly distributed. Allow from 8 to 12 hours for drying unless the process can be hastened by using an electric fan. In this procedure, place the slide film side up in front of an electric fan. Take particular care, however, in adjusting the distance from the fan so that the current of air will not pile the blood on one side of the film. Do not mail the slides until the films are entirely dry. Wrap each slide in soft paper or tissue and place them in the wooden boxes.

The sooner the specimens reach the laboratory after collection the better, since malaria parasites stain most characteristically in freshly prepared films.

Tissue For Microscopic Examination

In all districts of the state and especially in hospitals, there should be local approved laboratory service for pathology as well as other diagnostic examinations. If not available, steps should be taken to establish it.

The services rendered by competent resident pathologists are of invaluable assistance to surgeons in the study of their cases. Specimens of tissue therefore should be examined in a near-by approved laboratory. They should be sent to the Division of Laboratories and Research only when a question has arisen in regard to the diagnosis, when there is a difference of opinion concerning the findings, or when a confirmatory examination is desirable. Pertinent data relative to the clinical manifestations, operative findings, and treatment of the patient should accompany all specimens for histologic examination.

Tissues should be placed in fixative immediately after removal, since the most important step in their preparation is proper fixation. If this procedure is not followed, post-mortem changes may render the examination unsatisfactory. A 10-per-cent solution of ordinary commercial formalin is the best fixative for routine use; the volume should be at least ten times as great as that of the tissue. Whenever possible, all of the specimen that is removed, rather than a portion of it, should be sent to the laboratory for examination. Large specimens should be incised to permit proper penetration of the formalin, enclosed in jars, such as fruit jars, and shipped by express. Small specimens should be placed in a miscellaneous jar outfit with formalin, and sent by mail.

Occasionally, a cultural examination or an animal inoculation is desirable. Under such circumstances, some of the material should be collected on a swab or the specimen divided and a portion submitted in a sterile jar without preservative and the remainder in one containing formalin.

Fig. 90c.

DIRECTIONS FOR MAILING SPECIMENS

Before being mailed, all of the packing should be replaced so that the outfit will conform to the postal regulations. The cap of the mailing case should be screwed on securely. Specimens should be mailed as soon as possible after collection.

The diagnostic facilities of the Division of Laboratories and Research are designed primarily to render prompt assistance to health officers and physicians in the diagnosis and control of communicable diseases, and to provide laboratory service for all persons in the State for whom such service is not otherwise available.

Fig. 90d.

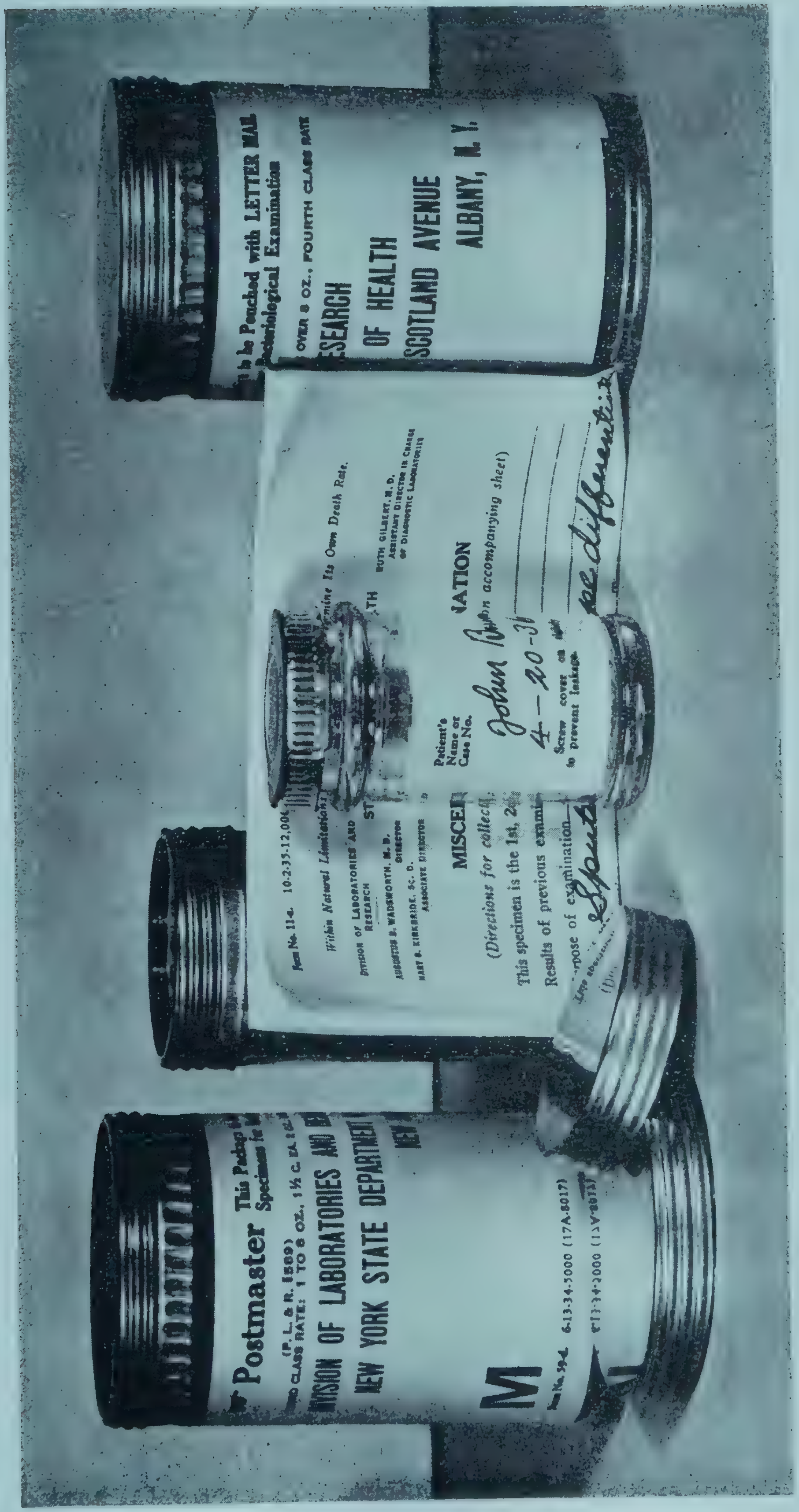

be Pouched with LETTER MAIL
Bacteriological Examination
OVER 8 OZ., FOURTH CLASS RATE
ESEARCH
OF HEALTH
SCOTLAND AVENUE
ALBANY, N. Y.
Form No. 114 10-2-35-12,000
Within Natural Limitations
mine Its Own Death Rate.
DIVISION OF LABORATORIES AND RESEARCH
AUGUSTUS B. WADSWORTH, M. D. DIRECTOR
MARY B. KIRKBRIDE, SC. D. ASSOCIATE DIRECTOR
RUTH GILBERT, M. D. ASSISTANT DIRECTOR IN CHARGE OF DIAGNOSTIC LABORATORIES
MISCE
ATION
(Directions for collect
on accompanying sheet)
This specimen is the 1st, 2nd
Results of previous exami
rpose of examination
Patient's Name or Case No.
John
4-20-36
Screw cover on tight to prevent leakage
Sputum
differentia
Postmaster This Package
Specimens for
(P. L. & R. 1559)
CLASS RATE: 1 TO 8 OZ., 1½ C. EA. 2 OZ.
DIVISION OF LABORATORIES AND RE
NEW YORK STATE DEPARTMENT
NEW
M
Form No. 39-d 6-13-34-5000 (17A-8017)

FIG. 91. JAR OUTFIT (1 oz.)

Jar Outfit (4 oz.)

Purpose.—Submission of sputum to be examined for tubercle bacilli—label, yellow, lettered "TB."

Mailing case, double.—*Outer container.* Fiber with threaded metal rim, 2¾ by 7 in. inside dimensions; corrugated paper pad for lining metal bottom and indented paper pad for metal screw cover. *Inside container.* Tin, 2½ by 6⅞ in. outside dimensions; corrugated paper pad lining and corrugated paper pad for lining bottom and felt pad for metal screw cover. Cellucotton for packing at top and bottom of jar inserted in inside container.

Jar.—Glass, 4 oz., round, wide-mouthed with metal screw cap. White printed label for identification of patient affixed.

Forms.—History form, 5 by 8 in., and directions for the collection of specimen folded and wrapped around inside containers. See figures 93a, 93b and 94. Also enclosed are directions for the protection of the worker (see fig. 92, p. 891).

Public Health Is Purchasable.
Within Natural Limitations Any Community Can Determine Its Own Death Rate.

NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY 1

EDWARD S. GODFREY, JR., M.D., *Commissioner*

DIVISION OF LABORATORIES AND RESEARCH

GILBERT DALLDORF, M.D.
Director

MARY B. KIRKBRIDE, SC.D.
Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

TUBERCULOSIS

EXAMINATION OF SPUTUM FOR TUBERCLE BACILLI ONLY

(*Directions for collecting specimen on reverse side*)

This specimen is the 1st, 2nd, 3d, etc..........

Dates on which specimen was collected..........

Purpose for which specimen was taken:

Diagnosis.......... To determine progress..........

Patient..........

Address..........
(St. or R. D.) (City, town or village) (County)

Occupation.......... Age.......... Sex.......... Color..........

Clinical diagnosis of tuberculosis: Definite.......... Suspected..........

Probably not tuberculosis..........

Clinical manifestations, x-ray findings, and family history:

....

....

....

....

Name and address of physician

....

....

Laboratory number.......... Received..........

Result of microscopic examination..........

Examined.......... Examiner..........

Result of cultural examination..........

Examined.......... Examiner..........

FIG. 93a. (Yellow) (Front)

Use local approved laboratory service, wherever available, for the examination of specimens.

CAUTION

Send only specimens of sputum to be examined for tubercle bacilli in these containers, as the specimens for this test are concentrated before they are examined and this procedure would render them unsatisfactory for other tests.

If any other types of examination are desired, send the specimens in similar containers with pink labels designed for specimens for miscellaneous examinations.

DIRECTIONS FOR THE COLLECTION OF MATERIAL

Collect sputum in the special jars designed for this purpose.

The first sputum coughed up in the morning is preferred. When the sputum is scanty, collect it for from twenty-four to seventy-two hours. If tubercle bacilli are not found, gastric lavage should be examined.

The tubercle bacilli are found most frequently during exacerbation of the disease and in purulent or cheesy sputum; less often, in pure mucus and blood. Following hemorrhage, select the purulent material for examination.

Do not fill the jar more than three-quarters full.

Be sure that the cap is fastened securely to avoid leakage.

Always indicate the identity of the patient on the label of the specimen jar.

Furnish the information requested and mail it with the specimen.

DIRECTIONS FOR MAILING SPECIMENS

Before being mailed, all of the packing should be replaced so that the outfit will conform to the postal regulations. The cap of the mailing case should be screwed on securely.

The diagnostic facilities of the Division of Laboratories and Research are designed primarily to render prompt assistance to health officers and physicians in the diagnosis and control of communicable diseases and to provide laboratory service for all persons in the State for whom such service is not otherwise available.

Specimen opened by.................................... Typed report sent ....

Specimen prepared by....................................

Preparation stained by....................................

Fig. 93b. (Yellow) (Back)

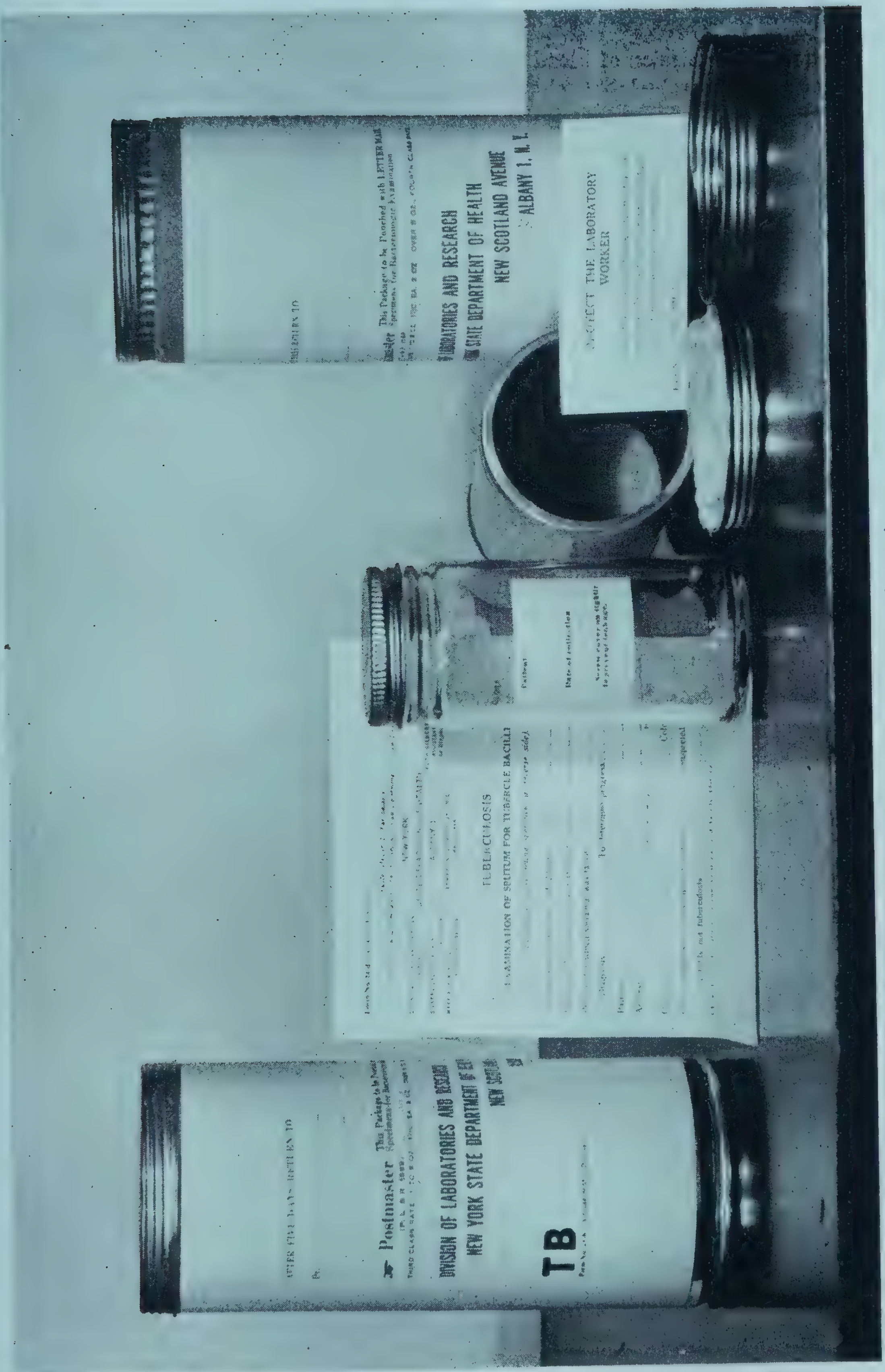

Postmaster
DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
NEW SCOTLAND AVENUE
ALBANY 1, N. Y.
TB
PROTECT THE LABORATORY WORKER
TUBERCULOSIS
EXAMINATION OF SPUTUM FOR TUBERCLE BACILLI

FIG. 94. JAR OUTFIT (4 oz.)

Tube Outfit with Needle

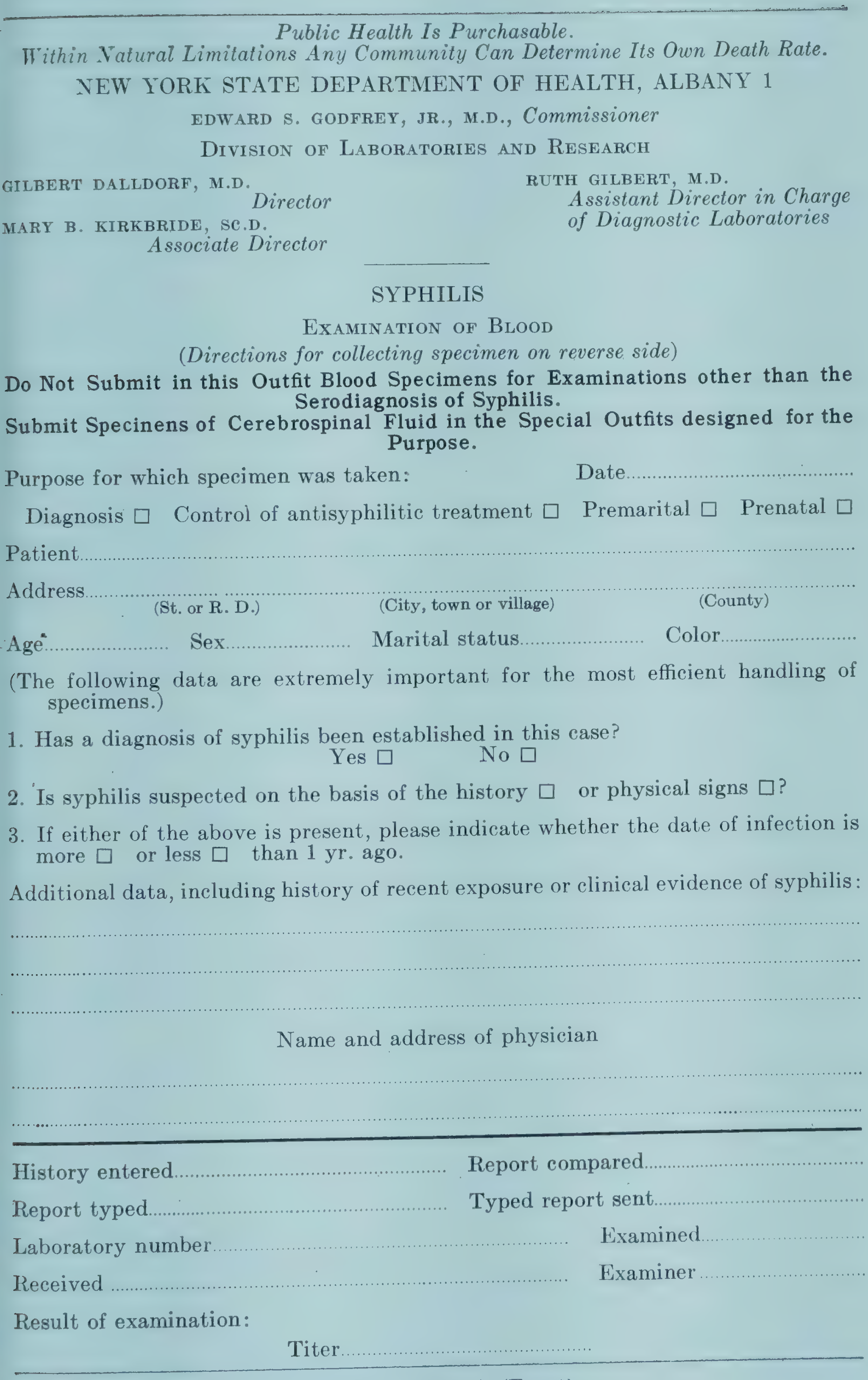
Purpose.—Submission of blood to be examined for evidence of: (1) Syphilis (complement-fixation test)—label, cherry or white with red printing, lettered "V" (see also "Cerebrospinal Fluid and Blood Outfit"); (2) Tuberculosis (complement-fixation test)—label, yellow, lettered "TB"; (3) Typhoid fever—label, Manila or white with green printing, lettered "T for agglutination test (Widal)."

Mailing case.—Fiber with threaded metal rim, 5$\frac{3}{4}$ in. outside height by 1$\frac{1}{16}$ in. inside diameter; corrugated paper lining and pads for lining metal bottom and metal screw cover. Cellucotton for packing placed at top and bottom of tube.

Test tube.—100 by 14$\frac{1}{2}$–16 mm. stoppered with No. 4$\frac{1}{2}$ or No. 5 cork, containing 19-gauge blood-letting needle (Petroff) 1$\frac{1}{2}$ in. long. White printed label for identification of patient affixed so that one end extends onto the cork.

Manila envelope.—1$\frac{1}{16}$ by 3$\frac{1}{4}$ in. for returning needle. Printed on envelope: "The blood-letting needle is valuable. After replacing stylet, enclose in envelope and return with specimen."

Forms.—History form, 5 by 8 in., and direction sheet with Manila envelope folded together and wrapped around test tube. See figures 95a, 95b; 96a, 96b; 97A, B, and C; and 86a–87b.



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NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY 1

EDWARD S. GODFREY, JR., M.D., *Commissioner*

DIVISION OF LABORATORIES AND RESEARCH

GILBERT DALLDORF, M.D.
Director
MARY B. KIRKBRIDE, SC.D.
Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

SYPHILIS

EXAMINATION OF BLOOD

(Directions for collecting specimen on reverse side)

Do Not Submit in this Outfit Blood Specimens for Examinations other than the Serodiagnosis of Syphilis.

Submit Specinens of Cerebrospinal Fluid in the Special Outfits designed for the Purpose.

Purpose for which specimen was taken: Date........................

Diagnosis ☐ Control of antisyphilitic treatment ☐ Premarital ☐ Prenatal ☐

Patient........................

Address........................ (St. or R. D.) (City, town or village) (County)

Age........ Sex........ Marital status........ Color........

(The following data are extremely important for the most efficient handling of specimens.)

1. Has a diagnosis of syphilis been established in this case? Yes ☐ No ☐
2. Is syphilis suspected on the basis of the history ☐ or physical signs ☐?
3. If either of the above is present, please indicate whether the date of infection is more ☐ or less ☐ than 1 yr. ago.

Additional data, including history of recent exposure or clinical evidence of syphilis:

....

....

....

Name and address of physician

....

....

History entered........................ Report compared........................

Report typed........................ Typed report sent........................

Laboratory number........................ Examined........................

Received........................ Examiner........................

Result of examination:

Titer........................

FIG. 95a. (White) (Front)

DIRECTIONS FOR COLLECTING AND MAILING SPECIMENS

Use local approved laboratory service, wherever available, for the examination of specimens.

Submit about 10 ml. of blood, collected with aseptic precautions three or four hours after eating, or, preferably, in the morning before breakfast.

Examination of specimens for evidence of syphilis when the patient is acutely ill with some other disease is not recommended. When specimens are collected under these conditions, pertinent data in regard to the clinical findings and diagnosis should be recorded on the history form.

Specimens collected with the sterile needles furnished in the outfits are usually found to be satisfactory for serologic tests provided aseptic precautions have been observed, unless during very cold weather the blood has been allowed to freeze. At such times, delivery of the specimens at a post office rather than leaving them in a mail box on the street is desirable.

If syringes are used to collect the blood, careful cleaning and sterilization in dry heat is the procedure of choice. If boiled, they should be rinsed with sterile physiologic salt solution and allowed to cool before use.

When the specimen is prepared for mailing, all of the packing should be replaced so that the outfit will conform to the postal regulations. The cap of the mailing case should be screwed on securely.

The diagnostic facilities of the Division of Laboratories and Research are designed primarily to render prompt assistance to health officers and physicians in the diagnosis and control of communicable diseases, and to provide laboratory service for all persons in the state for whom such service is not otherwise available.

FIG. 95b. (White) (Back)

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Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

TUBERCULOSIS

EXAMINATION OF BLOOD BY COMPLEMENT-FIXATION TEST

(Directions for collecting specimen on reverse side)

Patient.. Age....................
Address...
(St. or R. D.) (City, town or village) (County)
Occupation.................................... Sex....................................
Number of specimen 1, 2, 3, etc.................. Date taken....................
Pulmonary tuberculosis.............................. Result of x-ray....................
Extent of area involved.............................. Cavities....................
Active tuberculosis...
Lesions—infiltration: Slight.............................. Marked....................
Consolidation.............................. Tuberculous complications....................
Symptoms: Slight or none.............................. Moderate....................
Severe.............................. Impairment of function....................
Result of sputum examination.............................. Dates....................
Febrile reaction.............................. °F....................
Stage: Incipient.................. Moderately adv.................. Far adv..................
Inactive tuberculosis.............................. Constitutional symptoms and expectoration
with bacilli absent.............................. How long....................
Physical signs: Stationary.............................. Improved....................
Healed lesion.................. Retrogressive.................. How long..................
Nonpulmonary tuberculosis (organs or tissues involved)....................
....
....
Prognosis...
Family history...
....
....
....
Syphilis or other complicating infection present....................................
Record any additional clinical data on reverse side.

Name and address of physician

....
....

FIG. 96a. (Yellow) (Front)

DIRECTIONS FOR COLLECTING BLOOD

Submit about 10 ml. (cc.) of blood, collected with aseptic precautions three or four hours after eating, or, preferably, in the morning before breakfast.

Specimens collected with the sterile needles furnished in the outfits are usually found to be satisfactory for serologic tests provided aseptic precautions have been observed, unless during very cold weather the blood has been allowed to freeze. At such times, delivery of the specimens at a post office rather than leaving them in a mail box on the street is desirable.

If syringes are used to collect the blood, careful cleaning and sterilization in dry heat is the procedure of choice. If boiled, they should be rinsed with sterile physiologic salt solution and allowed to cool before they are used.

DIRECTIONS FOR MAILING SPECIMENS

Before being mailed, all of the packing should be replaced so that the outfit will conform to the postal regulations. The cap of the mailing case should be screwed on securely.

Record of additional clinical data:

Laboratory number.. Examined....................................

Received.. Examiner....................................

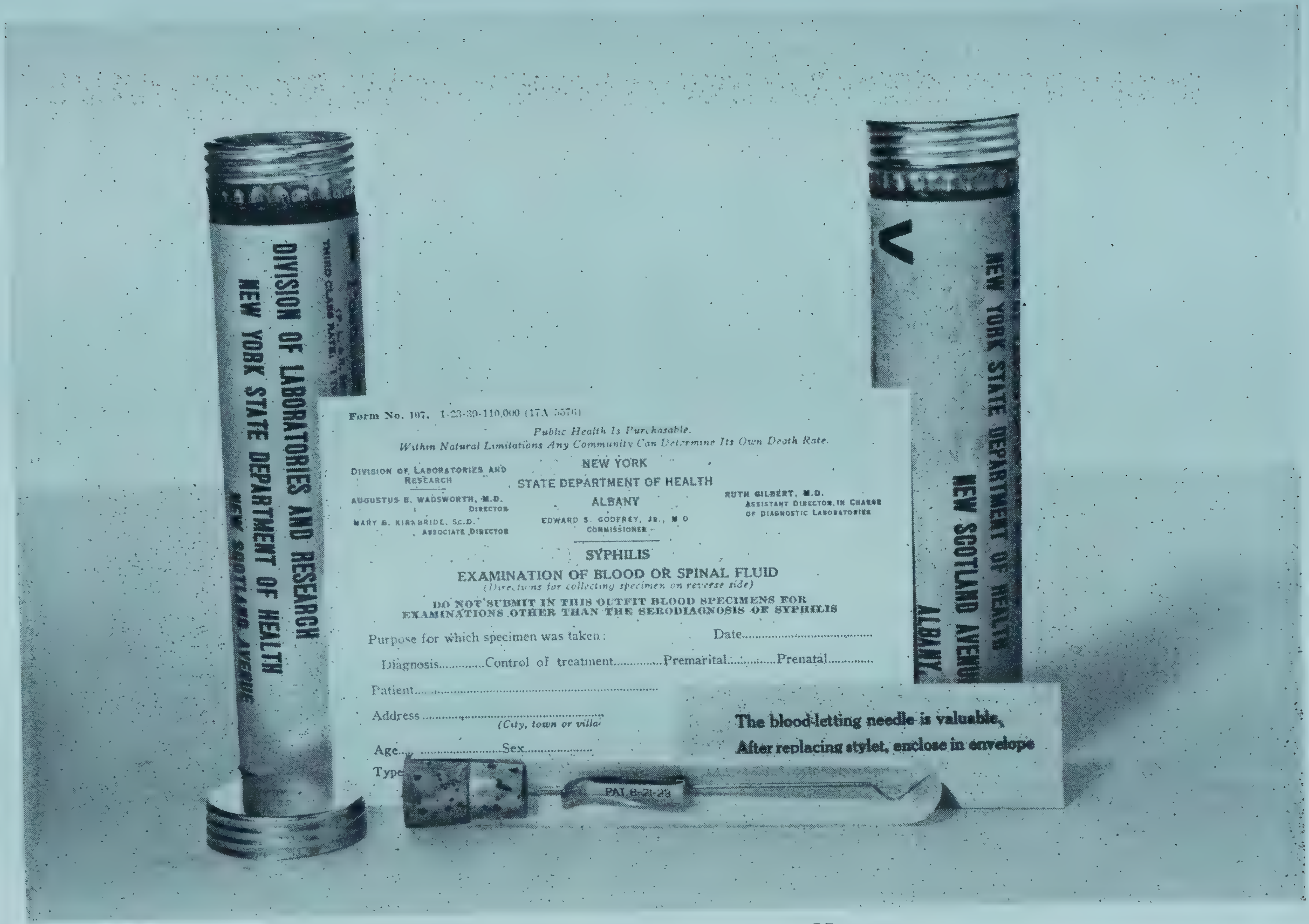
Result of examination: Titer..

Lab. No. (Syphilis)........................ Examined........................ Examiner........................

Result of test for syphilis...

Typed report sent on..

Fig. 96b. (Yellow) (Back)



THIRD CLASS RATE
DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
NEW SCOTLAND AVENUE
V
NEW YORK STATE DEPARTMENT OF HEALTH
NEW SCOTLAND AVENU
ALBANY
PAT. 8-21-23

Form No. 107. 1-23-30-110,000 (17A 5576)

Public Health Is Purchasable.
Within Natural Limitations Any Community Can Determine Its Own Death Rate.

NEW YORK
STATE DEPARTMENT OF HEALTH
ALBANY

DIVISION OF LABORATORIES AND RESEARCH
AUGUSTUS B. WADSWORTH, M.D.
DIRECTOR
MARY B. KIRKBRIDE, Sc.D.
ASSOCIATE DIRECTOR

EDWARD S. GODFREY, JR., M.D.
COMMISSIONER

RUTH GILBERT, M.D.
ASSISTANT DIRECTOR, IN CHARGE OF DIAGNOSTIC LABORATORIES

SYPHILIS

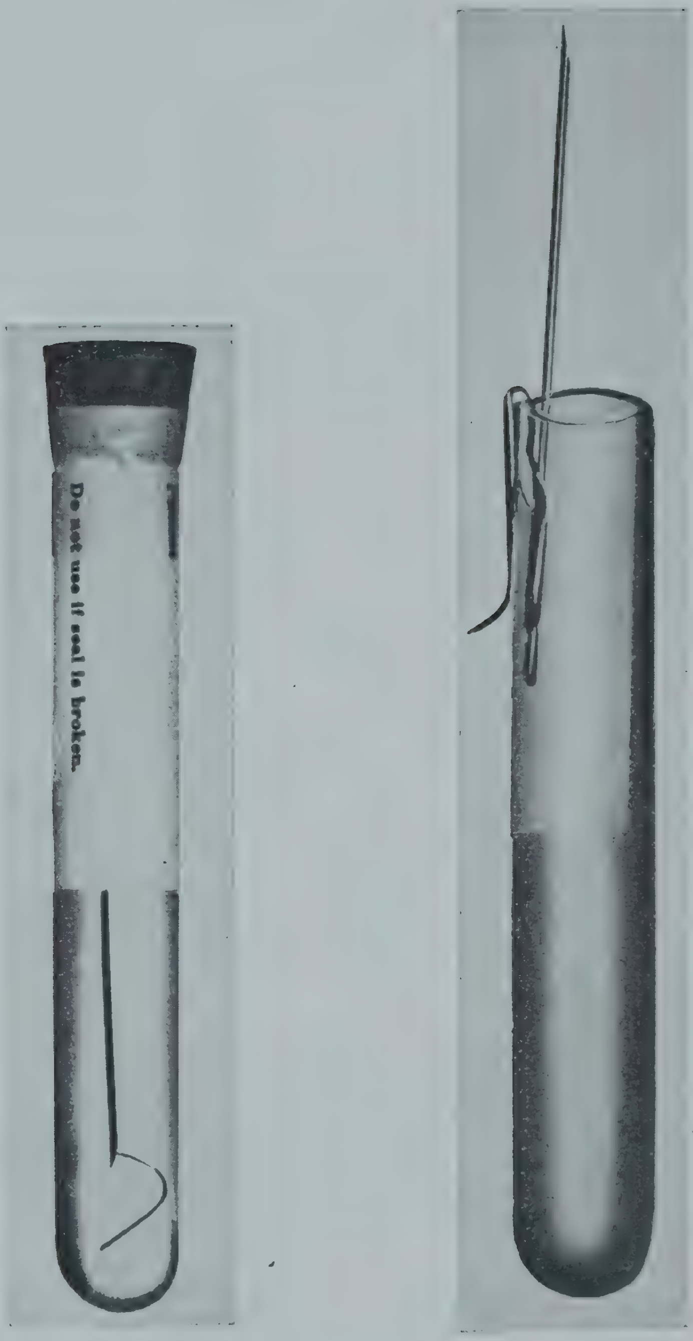
EXAMINATION OF BLOOD OR SPINAL FLUID
(Directions for collecting specimen on reverse side)

DO NOT SUBMIT IN THIS OUTFIT BLOOD SPECIMENS FOR EXAMINATIONS OTHER THAN THE SERODIAGNOSIS OF SYPHILIS

Purpose for which specimen was taken: Date..........
Diagnosis..........Control of treatment..........Premarital..........Prenatal..........
Patient..........
Address.......... *(City, town or villa*
Age..........Sex..........
Type

The blood-letting needle is valuable,
After replacing stylet, enclose in envelope

FIG. 97. A. TUBE OUTFIT WITH NEEDLE


Do not use if seal is broken.

B C

Fig. 97. Tube From Outfit With Needle
B. When Cork Is Removed, Label Is Broken
C. Attachment of Blood-Letting (Petroff) Needle When Specimen Is Collected

Cerebrospinal Fluid and Blood Outfit

Purpose.—Submission of cerebrospinal fluid and blood to be examined for evidence of syphilis of the central nervous system—label, cherry or white with red printing, marked "Spinal Fluid."

Mailing case.—Fiber with threaded metal rim, 5¾ by 2 in. inside dimensions; corrugated paper lining and pad for lining metal bottom and felt pad for metal screw cover. Kimpak crepe wadding, 10 ply, Kraft backed, punch embossed, 5 by 7½ in. wrapped around tubes. Cellucotton for packing placed at top and bottom of tubes.

Test tubes.—One pointed, 100 by 14.5–16 mm., for cerebrospinal fluid; one, round bottom, 100 by 14.5–16 mm., for blood specimen, containing 19-gauge blood-letting needle (Petroff); stoppered with No. 4½ or No. 5 paraffined corks. White printed label for identification of patient affixed so that one end extends onto the cork.

Manila envelope.—See "Tube Outfit with Needle"; placed around tubes inside Kimpak crepe wadding.

Forms.—History form, 5 x 8 in., and direction sheet rolled around test tubes and Manila envelope. See figures 98a, 98b and 99.

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Within Natural Limitations Any Community Can Determine Its Own Death Rate.

NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY 1

EDWARD S. GODFREY, JR., M.D., *Commissioner*

DIVISION OF LABORATORIES AND RESEARCH

GILBERT DALLDORF, M.D.
Director
MARY B. KIRKBRIDE, SC.D.
Associate Director

RUTH GILBERT, M.D.
Assistant Director in Charge of Diagnostic Laboratories

SYPHILIS

EXAMINATION OF CEREBROSPINAL FLUID

(Directions for collecting specimen on reverse side)

EVERY SPECIMEN OF CEREBROSPINAL FLUID SHOULD BE ACCOMPANIED BY A SPECIMEN OF THE PATIENT'S BLOOD

Do Not Send Specimens for Bacteriologic Examination in this Outfit

Purpose for which specimen was taken:

Diagnosis ☐ Control of antisyphilitic treatment ☐ Date

Patient

Address
(St. or R. D.) (City, town or village) (County)

Age Sex Marital Status Color

1. Has a diagnosis of syphilis been made? Yes ☐ No ☐

If yes, more ☐ or less ☐ than 4 years since infection?

2. Please list the signs and symptoms of neurologic disease, if any are present.

Name and address of physician

Laboratory number Examined
Received Examiner
Result of c. f. test:
Titer

FIG. 98a. (White) (Front)

DIRECTIONS FOR COLLECTING SPECIMENS

Use local approved laboratory service, wherever available, for the examination of specimens.

Collect a specimen of cerebrospinal fluid in the tube with the pointed tip and a specimen of blood in the one with the rounded base.

If a bacteriologic examination is desired, collect the specimens in the outfits designed for the test or in the containers with pink labels used for specimens for miscellaneous examinations.

Cerebrospinal Fluid

Proper collection of the specimen is of particular importance. The specimen should consist of at least 5 ml. of fluid.

When a lumbar puncture is made, two needles, thoroughly cleaned and sterile, should be available. They should have been carefully sharpened, since the use of a dull needle is usually responsible for admixture of blood in specimens of cerebrospinal fluid. If there is evidence of blood in the fluid, the tap should be discontinued and another puncture made with a fresh needle in the next interspace above the one that has been entered. Blood, oil, contaminating bacteria, or any other foreign material in the cerebrospinal fluid usually renders it unsatisfactory.

Under no circumstances should the cerebrospinal fluid be centrifuged and the sediment removed before the specimen is sent to the laboratory.

Blood

Submit about 10 ml. of blood, collected with aseptic precautions three or four hours after eating, or, preferably, in the morning before breakfast.

Examination of specimens for evidence of syphilis when the patient is acutely ill with some other disease is not recommended. When specimens are collected under these conditions, pertinent data in regard to the clinical findings and diagnosis should be recorded on the history slip.

Specimens collected with the sterile needles furnished in the outfits are usually found to be satisfactory for serologic tests provided aseptic precautions have been observed, unless during very cold weather the blood has been allowed to freeze. At such times, delivery of the specimens at a post office rather than leaving them in a mail box on the street is desirable.

If syringes are used to collect the blood, careful cleaning and sterilization in dry heat is the procedure of choice. If boiled, they should be rinsed with sterile physiologic salt solution and allowed to cool before they are used.

DIRECTIONS FOR MAILING SPECIMENS

Before being mailed, all of the packing should be replaced so that the outfit will conform to the postal regulations. The cap of the mailing case should be screwed on securely.

The diagnostic facilities of the Division of Laboratories and Research are designed primarily to render prompt assistance to health officers and physicians in the diagnosis and control of communicable diseases, and to provide laboratory service for all persons in the state for whom such service is not otherwise available.

Typed report sent on..

Fig. 98b. (White) (Back)

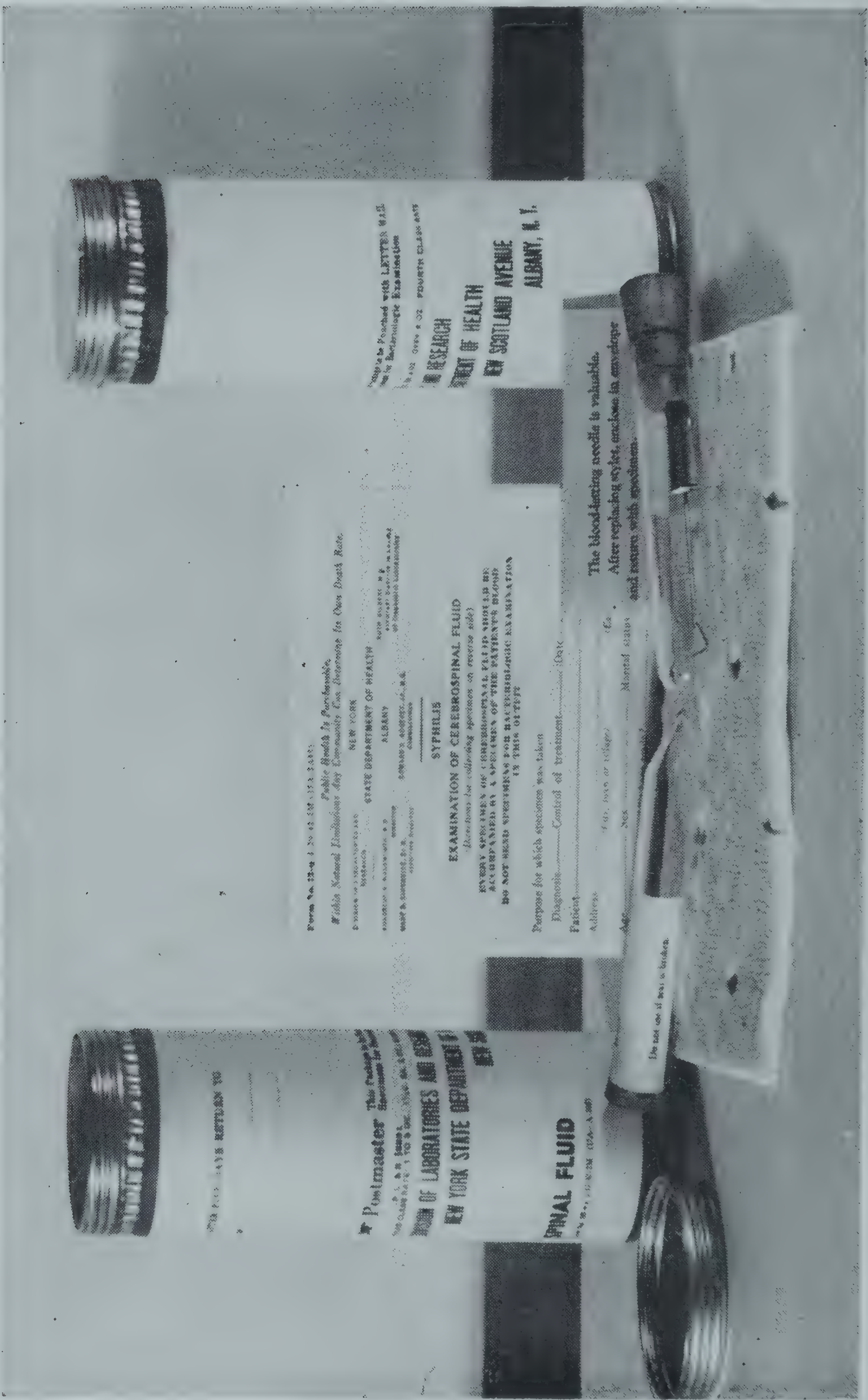
NEW SCOTLAND AVENUE
ALBANY, N.Y.
STATE DEPARTMENT OF HEALTH
EXAMINATION OF CEREBROSPINAL FLUID
SYPHILIS
The blood-letting needle is valuable.
After replacing stylet, enclose in envelope
and return with specimen.
Postmaster
NEW YORK STATE DEPARTMENT
PINAL FLUID

FIG. 99. CEREBROSPINAL FLUID AND BLOOD OUTFIT

Tube Outfit with Swab

Purpose.—Submission of exudate or fluid for bacteriologic examination—label, pink, lettered "M."

Mailing case, double.—See "Diphtheria Culture Outfit," p. 881.

Test tube.—100 by 14½–16 mm. with white printed label for identification of patient affixed so that one end extends onto the cork.

Swab.—Absorbent cotton on 16-gauge wire 3¾ in. long inserted in No. 4½ or No. 5 paraffined cork to stopper tube.

Forms.—History form, 5 by 8 in., and direction sheet folded and wrapped around tube. See figures 88, 90a, 90b, 90c, and 90d.

Chancre Fluid Outfit containing Capillary Tubes

Purpose.—Submission of specimens to be examined for *Treponema pallidum*—label, white, lettered "V."

Mailing case, double.—See "Diphtheria Culture Outfit," p. 881.

Test tube.—100 by 11.5–12.5 mm., with white printed label for identification of patient affixed so that one end extends onto the cork.

Capillary tubes.—3 vaccine capillary tubes, 68 mm. long, 1.05–1.2 mm. in diameter inserted in test tube stoppered with No. 2½ paraffined cork.

Vial.—1 ml., glass, one-half full of beeswax-vaseline mixture (*F80*); stoppered with No. 00 or No. 0 cork; held to test tube with rubber band.

Forms.—History form, 5 by 8 in., and direction sheet folded and wrapped around test tube and vial. See figures 100a, 100b and 101.

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NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY 1

EDWARD S. GODFREY, JR., M.D., *Commissioner*

DIVISION OF LABORATORIES AND RESEARCH

GILBERT DALLDORF, M.D. *Director*	RUTH GILBERT, M.D. *Assistant Director in Charge of Diagnostic Laboratories*
MARY B. KIRKBRIDE, SC.D. *Associate Director*	

SYPHILIS

EXAMINATION FOR TREPONEMA PALLIDUM BY DARK FIELD

(Directions for collecting specimen on reverse side)

Specimen taken from suspected chancre........ chancroid........ gland puncture..............

Date of collection..

Patient..

Address..
(St. or R. D.) (City, town or village) (County)

Age.................................... Sex.................................... Marital Status..

Duration of lesion.. Location..

Date exposed..

Has any local antiseptic or other treatment been administered?..

Has patient a history of gonorrhea?..

Clinical manifestations and family history:
(These data are extremely important guides to the number and kind of tests to be made)

....

....

....

....

....

Name and address of physician

....

....

Laboratory number.. Examined..

Received.. Examiner..

Result of examination..

FIG. 100a. (White) (Front)

DIRECTIONS FOR COLLECTING MATERIAL TO BE EXAMINED FOR TREPONEMA PALLIDUM

Use local approved laboratory service, wherever available, for the examination of specimens.

Collection of Specimens from Suspected Chancres and Chancroids

Wash the lesion with sterile physiologic salt solution. Rub firmly with sterile gauze (a compress of 2 per-cent novocaine applied for a few minutes will aid in obtaining the deep exudate). Remove blood with sterile gauze.

Gently compress the tissues at the base of the lesion until a drop of clear serum collects on the abraded surface. Touch this drop with the capillary tube, which is held in a horizontal position. The opposite end should be open. The serum or plasma will then rapidly enter the tube owing to capillary action. Seal by pressing each end of the tube into the wax in the amber-glass vial. While doing this, hold the tube in a horizontal position so that the serum will not run out.

Repeated tests are desirable as failure to demonstrate the spirochetes does not exclude syphilis.

If an antiseptic or other local treatment has been administered, a salt-solution compress can be applied and the patient instructed to return on successive days for the collection of specimens or, in case the regional glands are enlarged, a specimen may be collected in accordance with the directions which follow.

Certain of the spirochetes found in nonsyphilitic lesions of the mouth resemble *Treponema pallidum* so closely that the results of morphologic examinations of fluid which contains exudate from the mouth or throat are of questionable diagnostic significance. In circumstances such as this, provided *Treponema pallidum* cannot be found in fluid from an enlarged regional lymph node, a series of specimens of the patient's blood should be submitted for serologic tests for evidence of syphilis.

Collection of Material from Enlarged Regional Lymph Nodes

In case an antiseptic has been used on the lesion and the regional lymph glands are enlarged, fluid from them also may be submitted. In a bilateral adenitis there may be an associated chancroidal infection and one or more of the glands may be fluctuating, inflammatory and tender. The indurated, shotty, nontender glands should be chosen.

Sterilize the skin over the selected lymph node. Draw about 0.5 ml. of sterilized salt solution into a 1- or 2-ml. sterile syringe using a 22- or 24-gauge needle. Immobilize the gland by grasping it so that the skin is drawn tightly over it. Thrust the needle into the gland, rotate it to break apart some of the tissue at its tip, and inject a few drops of the salt solution, taking care that the point of the needle enters the gland and not the surrounding tissues. Draw into the syringe a few drops of the fluid from the glandular tissue.

The aspirated fluid (which should contain very little blood) may then be deposited from the syringe onto a clean glass surface such as that of a slide or the side of a flat bottle, and collected in capillary tubes in the same manner as described for fluid from a chancre.

Submit also a specimen of blood (about 10 ml.) for the complement-fixation test for syphilis in the outfit designed for the purpose.

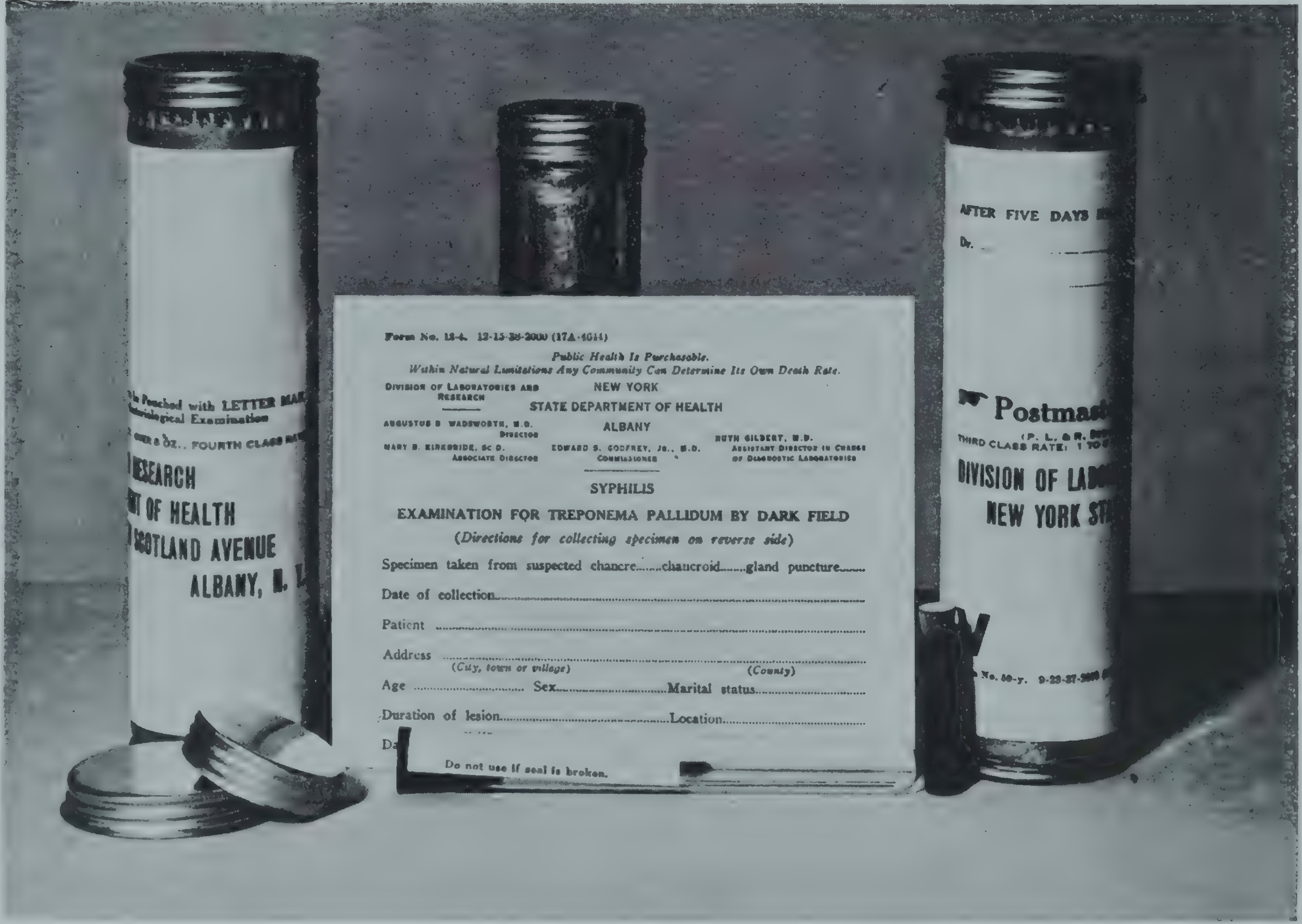
DIRECTIONS FOR MAILING SPECIMENS

Before mailing, all of the packing should be replaced so that the outfit will conform to the postal regulations. The cap of the mailing case should be screwed on securely. Specimens should be mailed as soon as possible after collection.

Specimen opened by..

Typed report sent on..

Fig. 100b. (White) (Back)



Form No. 13-4. 12-15-38-2000 (17A-4614)

Public Health Is Purchasable.
Within Natural Limitations Any Community Can Determine Its Own Death Rate.

Division of Laboratories and Research

NEW YORK
STATE DEPARTMENT OF HEALTH
ALBANY

Augustus B. Wadsworth, M.D.
Director

Mary B. Kirkbride, Sc.D.
Associate Director

Edward S. Godfrey, Jr., M.D.
Commissioner

Ruth Gilbert, M.D.
Assistant Director in Charge of Diagnostic Laboratories

SYPHILIS

EXAMINATION FOR TREPONEMA PALLIDUM BY DARK FIELD

(Directions for collecting specimen on reverse side)

Specimen taken from suspected chancre........chancroid........gland puncture........

Date of collection..........

Patient ....

Address .... (City, town or village) .... (County)

Age .... Sex.......... Marital status..........

Duration of lesion.......... Location..........

Do not use if seal is broken.

Punched with LETTER MA
teriological Examination
OVER 8 OZ., FOURTH CLASS RA
RESEARCH
T OF HEALTH
SCOTLAND AVENUE
ALBANY, N. Y.
AFTER FIVE DAYS
Dr.
Postmast
(P. L. & R.
THIRD CLASS RATE: 1 TO
DIVISION OF LAB
NEW YORK ST
No. 50-y. 9-23-37-

FIG. 101. CHANCRE FLUID OUTFIT CONTAINING CAPILLARY TUBES

Slide Outfit

Purpose.—Submission of films for microscopic examination for: (1) Gonococci—Manila envelope, lettered "V" (see also "Gonorrhea Culture Outfit"); (2) Other microscopic examinations—Manila envelope, lettered "M."

Mailing case.—Manila envelope, $4\frac{5}{8}$ by $6\frac{3}{4}$ in.

Slides.—2, glass, 3 by 1 in., etched $\frac{1}{2}$ in. on one end with Etch-O-Lite; enclosed in wooden box $3\frac{1}{8}$ by $1\frac{5}{16}$ by $\frac{9}{16}$ in.; printed directions secured to the box with cord. Each slide in outfit marked "M" wrapped in 4 by 5 in. tissue paper.

Forms.—History form, 5 by 8 in., and directions for collecting specimens enclosed. See figures 82, 83a, and 83b for specimens to be examined for gonococci, and figures 88, 90a, 90b, 90c, and 90d for other microscopic examinations.

Rabies Outfit

NOT MAILABLE; MUST BE SENT BY EXPRESS

Purpose.—Submission of animal head to be examined for evidence of rabies—Manila tag envelope.

Shipping container.—Corrugated can, 24 gauge, galvanized sheet steel with side drop handles; diameter, top and bottom, $13\frac{1}{2}$ in., height $14\frac{1}{2}$ in.; capacity, $8\frac{1}{2}$ gal. Cover of same material, held tightly closed by two hasps fitted with key rings. Inside of can and cover sprayed with aluminum paint to prevent rusting. Excelsior for packing at top, bottom, and around sides of pail inserted in can.

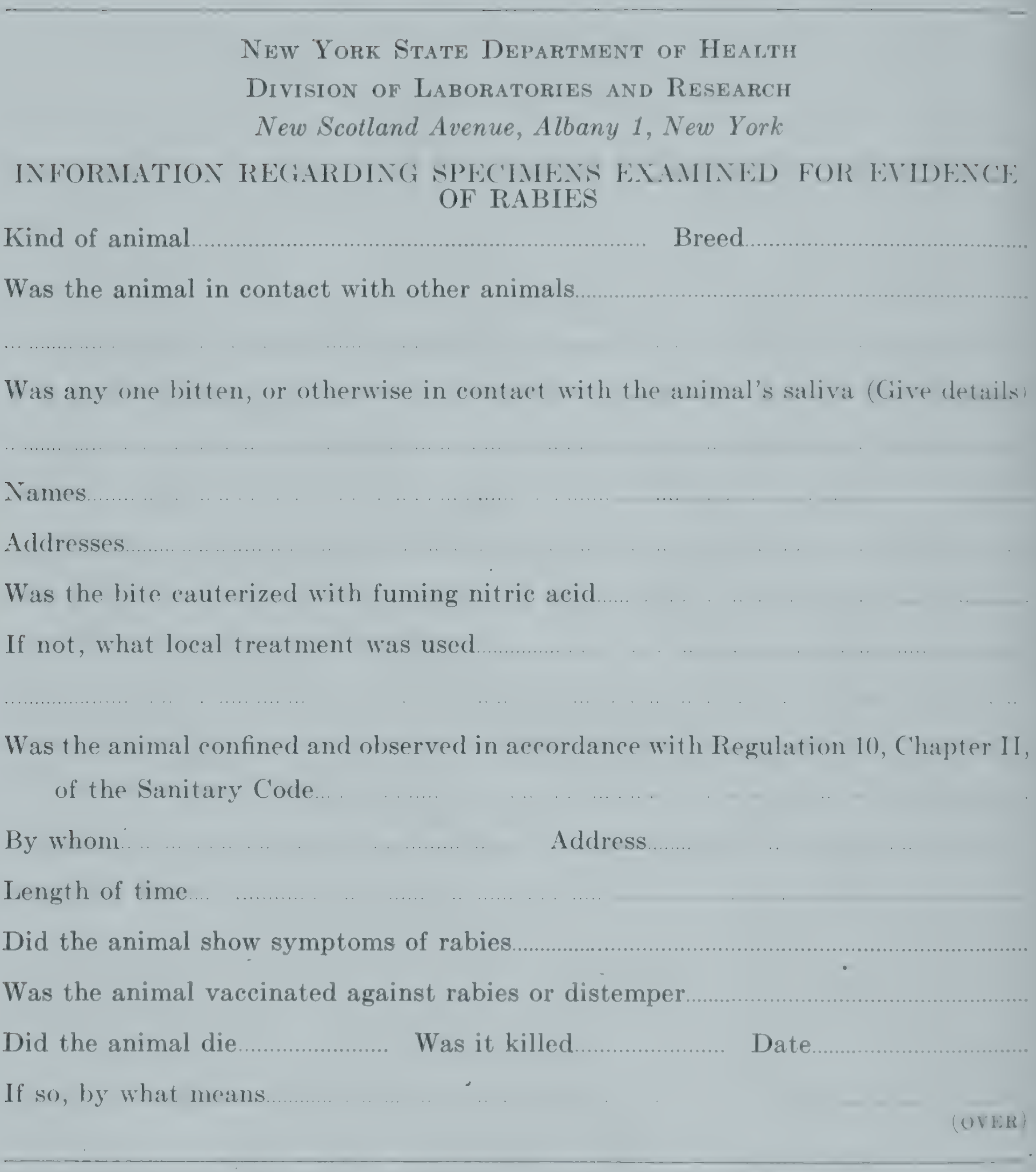
Pail.—Tin with or without wire bail handle, friction type cover flush with edge of pail; diameter, $8\frac{1}{4}$ in., height $9\frac{1}{2}$ in. White mimeographed label affixed on cover:

DIRECTIONS
AND
ADDRESSED ENVELOPE
ARE ENCLOSED

FIG. 102

Envelope.—Manila tag envelope (eyelet in one end with twine for attaching to shipping container), $4\frac{5}{8}$ by $6\frac{3}{4}$ in., marked "Perishable" on front and back.

Forms.—History form and direction sheet, white, each 5 by 8 in., enclosed in envelope and placed in the pail. See figures 103a, 103b, 104 and 105.



New York State Department of Health
Division of Laboratories and Research
New Scotland Avenue, Albany 1, New York

INFORMATION REGARDING SPECIMENS EXAMINED FOR EVIDENCE OF RABIES

Kind of animal.................................... Breed..............................

Was the animal in contact with other animals..

....

Was any one bitten, or otherwise in contact with the animal's saliva (Give details)

....

Names..

Addresses..

Was the bite cauterized with fuming nitric acid..

If not, what local treatment was used...

....

Was the animal confined and observed in accordance with Regulation 10, Chapter II, of the Sanitary Code...

By whom.................................... Address..

Length of time...

Did the animal show symptoms of rabies...

Was the animal vaccinated against rabies or distemper...

Did the animal die.................. Was it killed.................. Date..................

If so, by what means...

(OVER)

FIG. 103a. (White) (Front)

Name of owner..

Address..

Specimen submitted by..

Address..

Name of Health Officer...

Address..

Miscellaneous number.. Examined....................................

Received... Examiner....................................

Result of examination...

Typed report sent on..

Fig. 103b. (White) (Back)

New York State Department of Health

Division of Laboratories and Research

New Scotland Avenue, Albany 1, New York

DIRECTIONS FOR THE SUBMISSION OF ANIMALS' HEADS TO BE EXAMINED FOR EVIDENCE OF RABIES

Place the animal's head in the inside container and pound the cover down tightly. Then place the container with the specimen in the larger container and fill the space with cracked ice. (Dry ice should not be used.) Make certain that the cover of the outside container is fastened securely after the specimen has been packed.

Complete the history form, supplying the data requested, place it in the envelope bearing the address of the Division of Laboratories and Research, and tie the envelope securely to the handle on the outside container. Send the specimen to the laboratory by messenger if possible. If this cannot be done, ship it by express.

Fig. 104. (White)

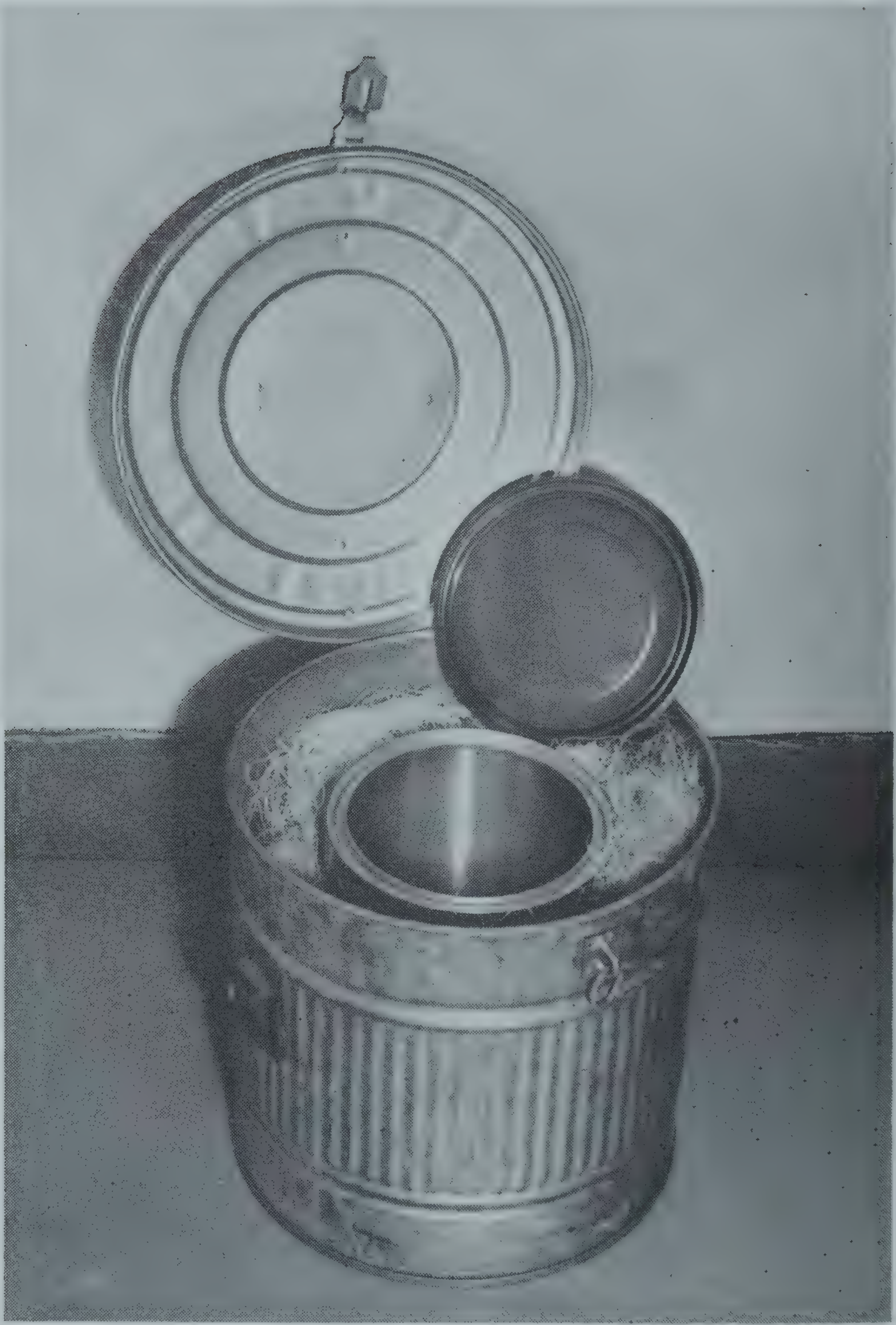


FIG. 105. RABIES OUTFIT

SAMPLES OF WATER

Bacteriologic Examination

A. Purpose.—The routine sampling of public water supplies.

Mailing case.—3-piece corrugated carton with seams reinforced with tape, 5½ in. long by 2⅜ in. square, outside dimensions; 1 piece wadding, Kimpak crepe, 10-ply Kraft backed, punched embossed, 6 by 7¾ in., in bottom of case.

Bottle.—4-oz., NoSolvit glass, extra wide mouth, with hood stopper; total height, 4¼ in., overall height, with stopper, 4$\frac{9}{16}$ in., width of mouth 1⅝ in. Stopper covered with either (a) unprinted, unparaffined paper cap for milk bottle, Standard Cap and Seal Corporation "J" die, tied in place; or (b) metal foil covered with unbleached muslin, grade B, each 4½ in. in diameter; tied in place with string. Label, Dennison No. 205 pasted on muslin.

Forms.—Card, 5 by 8 in., for information in regard to public water supplies and return address label. Printed on label: Fourth Class (P. L. and R. §574 and 590); card and label rolled in two pieces of Kimpak crepe wadding around bottle. See figures 106a and 106b.

B. Purpose.—For specimens other than those collected in the routine sampling of public water supplies.

Express case.—Box, wooden, with L-shape spring compressor in cover, 8¾ in. square by 8⅜ in. deep.

Ice can.—90 oz., square, round cornered, with screw cover and inner friction cap; tin, all plain gold lacquer outside; 5 by 5 by 7$\frac{7}{16}$ in. overall height.

Bottles.—5, 4-oz. NoSolvit glass (see Bottle, Outfit A) enclosed in fiber container with threaded metal rim, 2½ by 5¼ in. inside dimensions; indented paper pads for lining metal screw cover and corrugated paper pads for metal bottom.

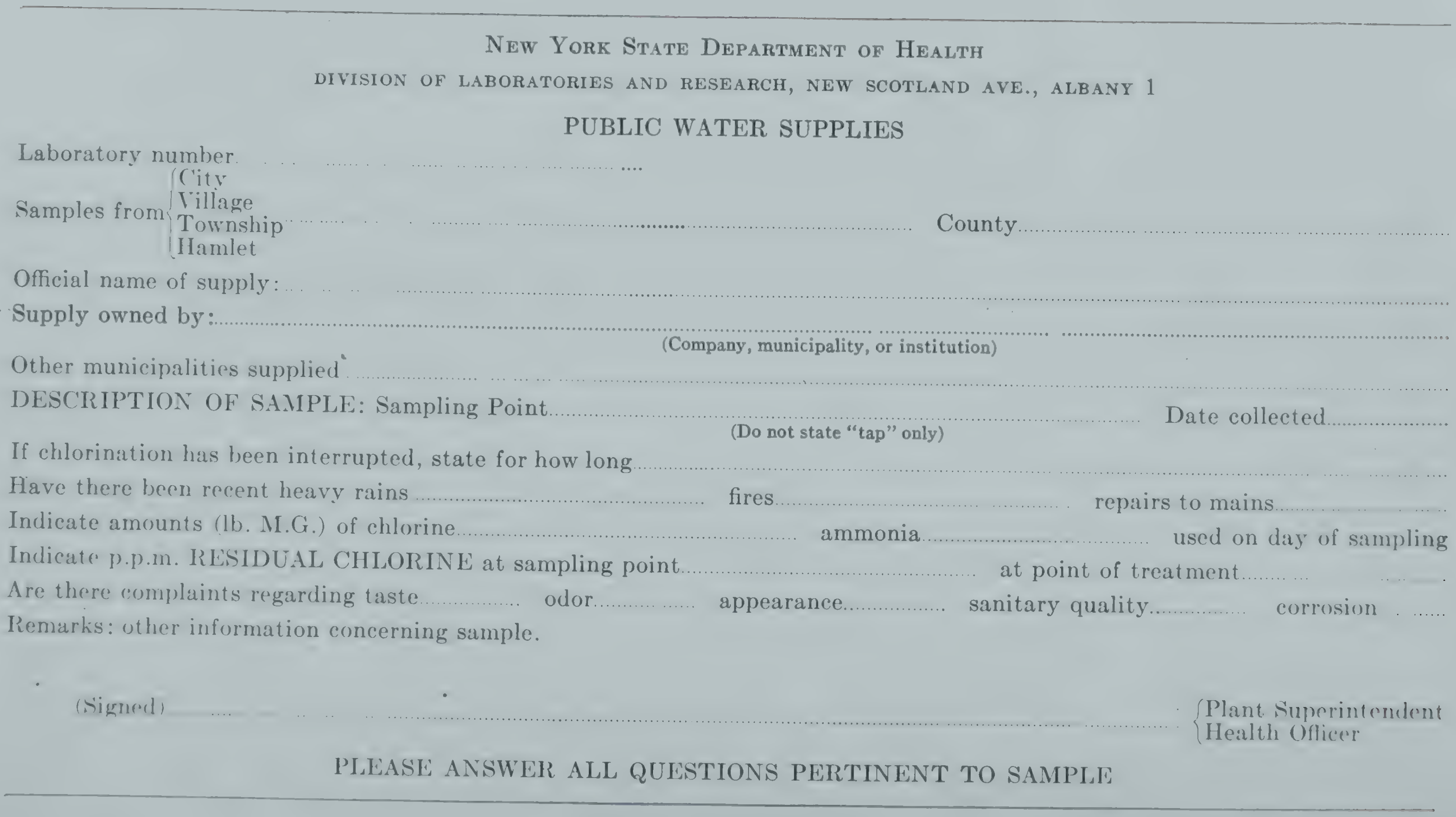
Forms.—Three printed information cards, 5 by 8 in.; printed tag with return address on one side and "Perishable" on the other enclosed in Manila envelope, 6½ by 9½ in. See figures 107, 108, and 109.

Chemical Examination

Express case.—Box, wooden, with spring compressors in corners, 7$\frac{1}{16}$ in. square by 16 in. deep, inside dimensions.

Bottle.—1 gal., NoSolvit glass, with hood stopper, 14¼ in. high by 5⅝ in. in diameter. Stopper covered with 8 in. square unbleached muslin, grade B, tied in place with string. Label, Dennison No. 205 pasted on muslin. Manila tag, Dennison No. 1G tied around neck of bottle; printed on tag: RINSE THOROUGHLY WITH WATER TO BE SAMPLED.

Forms.—Identification card, 5 by 8 in., and printed tag with return address on one side and "Perishable" on the other enclosed in Manila envelope 6½ by 9½ in. If not accompanied by outfit for bacteriologic examination, forms shown in figures 107, 108, and 109 are also enclosed.



NEW YORK STATE DEPARTMENT OF HEALTH

DIVISION OF LABORATORIES AND RESEARCH, NEW SCOTLAND AVE., ALBANY 1

PUBLIC WATER SUPPLIES

Laboratory number..............................

Samples from {City / Village / Township / Hamlet}.............................. County..............................

Official name of supply:..............................

Supply owned by:..............................
(Company, municipality, or institution)

Other municipalities supplied..............................

DESCRIPTION OF SAMPLE: Sampling Point.............................. Date collected..............................
(Do not state "tap" only)

If chlorination has been interrupted, state for how long..............................

Have there been recent heavy rains.............................. fires.............................. repairs to mains..............................

Indicate amounts (lb. M.G.) of chlorine.............................. ammonia.............................. used on day of sampling

Indicate p.p.m. RESIDUAL CHLORINE at sampling point.............................. at point of treatment..............................

Are there complaints regarding taste.............. odor.............. appearance.............. sanitary quality.............. corrosion..............

Remarks: other information concerning sample.

(Signed).............................. {Plant Superintendent / Health Officer}

PLEASE ANSWER ALL QUESTIONS PERTINENT TO SAMPLE

FIG. 106a. (Buff) (Front)

DO NOT WRITE ON THIS SIDE OF CARD

BACTERIAL EXAMINATION

Laboratory No.................................. Entered by.. Rec'd.............................. Exam...

Bacteria per ml.: agar 37° C—24 hrs.................................. Coliform group: Most Probable Number per 100 ml..

Agar Count 24 hrs.—37°C.		Vol.	Per Cent gas Lactose Broth		Endo or eosin methylene blue agar colonies	Ferm.	Gram Stain	Spores	Lactose Broth to Brilliant Green		Endo or eosin methylene blue agar colonies	Ferm.	Gram Stain	Spores
1 ml.	0.1 ml.		24 hrs.	48 hrs.					24 hrs.	48 hrs.				
		10 ml.												
		1 ml.												
CHLORIDES.....................p.p.m.		0.1 ml.												
NITRATES.......................p.p.m.														

Total Tests................ Reported to............................ Date.................... Compared by.................... Report sent..

FIG. 106b. (Buff) (Back)

NEW YORK STATE DEPARTMENT OF HEALTH

DIVISION OF LABORATORIES AND RESEARCH, NEW SCOTLAND AVE , ALBANY 1

GROUND WATERS

(Submit a card for each bottle filled)

Laboratory Number .... Date collected .... Date received in laboratory ....

Samples from (Check which) {City / Village / Hamlet / Township} .... County ....

Owner of water supply .... Tenant of property ....

DESCRIPTION OF SAMPLE {Chemical / Bacteriologic} : Sampling point ....

....

WELL: Dug .... Drilled .... Driven .... Depth of well .... Diameter ....

Type of cover .... Curb .... Casing .... Depth of casing .... Method of drawing water ....

SPRING: Location: Steep slope .... Level ground .... Type of cover .... Type of curb

Can surplus water pumped or surface drainage enter well or spring .... Recent {repairs .... / construction ....}

State geologic character of surrounding soil ....

State distance from all possible sources of pollution ....

(Include privies, cesspools, septic tanks, tile fields, barnyards, fertilized areas)

....

Is water supply involved in reported illness .... Explain ....

Signed .... Title ....

PLEASE ANSWER ALL QUESTIONS PERTINENT TO SAMPLE

FIG. 107. (Green) (Front)

Back of card same as Fig. 106b.

NEW YORK STATE DEPARTMENT OF HEALTH

DIVISION OF LABORATORIES AND RESEARCH, NEW SCOTLAND AVE., ALBANY 1

RESORT WATER SUPPLY

(Submit a card for each bottle filled)

Laboratory Number.................... Date collected.................... Date received in laboratory....................

Sample from **(Check which)** {City / Village / Hamlet / Township} .... County....................

Name of resort.................... Type of resort*....................

DESCRIPTION OF SAMPLE {Chemical / Bacteriologic} : Sampling point....................

....

Source of sample: Dug well.......... Driven well.......... Spring.......... Surface.......... Public supply..........
(Check description that applies)

Detailed identification of source....................

....

Supply chlorinated when sampled: Yes No Residual chlorine....................
(Check yes or no)

Sources of pollution....................

....

Current or recent illness connected with supply: Yes No Type of illness....................
(Check yes or no)

Date of last sample.................... Special tests required....................

Collected by.................... Title....................

* Use one and only one of the following designations: general camp, tourist camp, farm labor camp, other labor camp, hotel, boarding house, lodging house, tourist home.

PRINT LEGIBLY. COMPLETE ALL ITEMS

FIG. 108. (Pink) (Front)
Back of card same as Fig. 106b

NEW YORK STATE DEPARTMENT OF HEALTH

DIVISION OF LABORATORIES AND RESEARCH, NEW SCOTLAND AVE., ALBANY 1

WATER SUPPLY INSPECTION

Laboratory Number .... Date collected .... Date received in laboratory ....

Samples from (Check which) {City / Village / Hamlet / Township} .... County .... Public supply .... Private supply ....

Source of raw water .... Name of supply ....

Owner of supply .... Other municipalities served ....

DESCRIPTION OF SAMPLE {Chemical / Bacteriologic} : Sampling point ....

.... Routine / Special* ....

Treatment of supply: Prechlorination .... lb./m.g. Postchlorination .... lb./m.g. Ammonia .... lb./m.g.

Coagulants: .... lb./m.g. .... lb./m.g. .... lb./m.g.

Sedimentation: Time .... hrs. Filtration: Rapid sand .... Slow sand .... Filter runs .... hrs.

Residual chlorine in p.p.m.: Final effluent—Free .... Combined .... Tap—Free .... Combined ....

Volume of water treated .... m.g.d. Other types of treatment ....

Treatment problems:* Algae .... Taste .... Odor .... Appearance .... Corrosion .... Coagulation .... Chlorination ....

Indicate special tests required* ....

....

Other pertinent data ....

Signed .... Title ....

* Give details of problem and treatment on separate card or memorandum and *include with sample.*

FIG. 109 (White) (Front)
Back of card same as Fig. 106b.

INDEX

For convenience in reference, the names of media and auxiliary preparations and of stains and reagents are listed alphabetically by title under the headings Media, Media—Auxiliary preparations, and Stains and reagents. Reagents also appear in alphabetic order throughout the index, as well as all solutions.

Formulas for media and auxiliary preparations are indicated by bold-face italics; those for stains and reagents by bold-face roman type.

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